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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/083,058	02/25/2002	Svend Havclund	5386.224-US	6987

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New York, NY 10174-6401

EXAMINER

GUPTA, ANISH

ART UNIT	PAPER NUMBER
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1654

DATE MAILED: 05/27/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 10/083,058	Applicant(s) HAVELUND ET AL.	
	Examiner Anish Gupta	Art Unit 1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☐ Responsive to communication(s) filed on ____.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☐ Claim(s) 61-69 is/are pending in the application.
- 4a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) ____ is/are allowed.
- 6) ☐ Claim(s) 61-69 is/are rejected.
- 7) ☐ Claim(s) ____ is/are objected to.
- 8) ☐ Claim(s) ____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. ____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|--|---|
| 1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892) | 4) <input type="checkbox"/> Interview Summary (PTO-413)
Paper No(s)/Mail Date. ____. |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) | 5) <input type="checkbox"/> Notice of Informal Patent Application (PTO-152) |
| 3) <input type="checkbox"/> Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date ____. | 6) <input type="checkbox"/> Other: ____. |

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DETAILED ACTION

The preliminary amendment filed 2-25-02 is acknowledged. Claims 1-60 were cancelled and claims 61-69 were added. Claims 61-69 are pending in this application.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

The changes made to 35 U.S.C. 102(e) by the American Inventors Protection Act of 1999 (AIPA) and the Intellectual Property and High Technology Technical Amendments Act of 2002 do not apply when the reference is a U.S. patent resulting directly or indirectly from an international application filed before November 29, 2000. Therefore, the prior art date of the reference is determined under 35 U.S.C. 102(e) prior to the amendment by the AIPA (pre-AIPA 35 U.S.C. 102(e)).

1. Claims 61-69 are rejected under 35 U.S.C. 102(e) as being anticipated by Havelund et al.

The claims are drawn to water soluble aggregate of an insulin derivative having a lipophilic group and has a molecular weight larger than aldolase and comprise at least 2 zinc ions per 6 moles of insulin derivative.

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The reference teaches a composition comprising 600 nmol/ml of Lys B29-Ne-(hexadecanoyl)-insulin, 7 mM of sodium phosphate buffer at pH 7.5, 10 mM sodium chloride, 16 mM phenol, 16 mM cresol, 2-3 Zn²⁺/hexamer and 1.6%(w/v) glycerol (see col. 32, lines 34-48). The reference also discloses similar pharmaceutical formulations for Lys B29-Ne lithocholyl human insulin (see col. 31 and 32, lines 54-67 and 19-31). Note that this composition is similar to the composition as disclosed in the specification on page 13, line 28-29. Therefore, since the reference discloses the same composition as disclosed in the specification, with the same ionic strength and pH, the composition described in Havelund et al. would inherently result in aggregate formations.

2. Claims 61-69 are rejected under 35 U.S.C. 102(e) as being anticipated by Norup et al.

The claims are drawn to water soluble aggregate of an insulin derivative having a lipophilic group and has a molecular weight larger than aldolase and comprise at least 2 zinc ions per 6 moles of insulin derivative.

The reference teaches various insulin formulations that comprise insulin, phenolic compound such as cresol, glycerol, sodium chloride and varying amounts of zinc (see col. 4, lines 1-16 and 31-61). The pH of the composition is of the 7.2 when 20 mM of NaCl is present (see col. 4, lines 34-62). The composition utilizes insulin derivatives that include, B29-Ne-(N-lithocholyl - γ -glutamyl)-des(B30)-human insulin (see col. 3, lines 499-61). The difference between the prior art and the instant application is that the reference does not specifically teach aggregation of the insulin. However, since the reference discloses a composition with similar ionic strength and pH as the claimed composition, the composition disclosed by the reference would necessarily result in aggregate formations. Moreover, since the reference teaches pharmaceutical formulations that are

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intended to be used in-vivo, the formations aggregates would have occurred after injections since the environment would have the ionic strength and pH necessary for aggregates to form. Note the claims state that the aggregates are "formed in an environment having an ionic strength and pH of the tissue **after** subcutaneous injections."

3. Claims 61-69 are rejected under 35 U.S.C. 102(b) as being unpatentable over Havelund et al. (WO 95/07931).


The claims are drawn to water soluble aggregate of an insulin derivative having a lipophilic group and has a molecular weight larger than aldolase and comprise at least 2 zinc ions per 6 moles of insulin derivative.

The reference teaches insulin composition comprising 600 nmol/ml of insulin, 7 mM of sodium phosphate buffer at pH 7.5, 10 mM sodium chloride, 16 mM phenol, 16 mM cresol, 2-3 Zn²⁺/hexamer and 1.6%(w/v) glycerol (see page 55-56). For insulin analogs, the reference teaches, as acknowledged by Applicants on page 10 of the specification, the use of NeB29-lipochoyl- α -glutamyl des (B30) (see page 54, lines 13-25). Furthermore, the reference states that the parenteral administration may be performed by subcutaneous injection (see page 27, lines 8-10). Therefore, since the reference discloses the same composition as disclosed in the specification, with the same ionic strength and pH, the composition described in Havelund et al. would necessarily result in aggregate formations. Thus since all of the structural limitations of the compound are met and the same mode of administration is also disclosed, the aggregation of the compound, after injection, would be necessarily achieved.

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4. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anish Gupta whose telephone number is (571)272-0965. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback , can normally be reached on (571) 272-0961. The fax phone number of this group is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

 5/18/04
Anish Gupta
Patent Examiner

Notice of References Cited	Application/Control No. 10/083,058	Applicant(s)/Patent Under Reexamination HAVELUND ET AL.	
	Examiner Anish Gupta	Art Unit 1654	Page 1 of 1

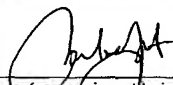
U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-5,866,538	02-1999	Norup et al.	514/3
	B	US-6,011,007	01-2000	Havelund et al.	514/3
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO95/07931	03-1995	PCT	Havelund et al.	
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	 5/19/04

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C07K 14/62, A61K 38/28	A1	(11) International Publication Number: WO 95/07931 (43) International Publication Date: 23 March 1995 (23.03.95)
(21) International Application Number: PCT/DK94/00347 (22) International Filing Date: 16 September 1994 (16.09.94) (30) Priority Data: 1044/93 17 September 1993 (17.09.93) DK 08/190,829 2 February 1994 (02.02.94) US (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): HAVELUND, Svend [DK/DK]; Kurvej 24, DK-2880 Bagsvaerd (DK). HALSTRØM, John, Broberg [DK/DK]; Søndergade 44, DK-3390 Hundested (DK). JONASSEN, Ib [DK/DK]; Valby Langgade 10, DK-2500 Valby (DK). ANDERSEN, Asser, Sloth [DK/DK]; Grundtvigsvej 35, 2.tv., DK-1864 Frederiksberg C (DK). MARKUSSEN, Jan [DK/DK]; Kikudbakken 7, DK-2730 Herlev (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD). Published <i>With international search report.</i>
(54) Title: ACYLATED INSULIN (57) Abstract <p>The present invention relates to protracted human insulin derivatives in which the A21 and the B3 amino acid residues are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the B30 amino acid residue is a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ε-amino group of Lys^{B29}; or b) the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in any of which cases the ε-amino group of Lys^{B29} has a lipophilic substituent; and any Zn²⁺ complexes thereof with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is present, then the insulin derivative is always present as a Zn²⁺ complex.</p>		

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ACYLATED INSULIN**FIELD OF THE INVENTION**

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the requirement related to meals.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be

immunogenic in man and that their use for medical purposes may lead to formation of antibodies (Samuel et al., Studies on the immunogenecity of protamines in humans and experimental animals by means of a micro-complement fixation test, Clin. Exp. Immunol. 33, pp. 252-260 (1978)).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz et al., Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologica 25, pp. 322-324 (1983)). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback with these solutions is that the particle size distribution of the precipitate formed in the tissue on injection, and thus the timing of the medication, depends on the blood flow at the injection site and other parameters in a somewhat unpredictable manner. A further drawback is that the solid particles of the insulin may act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction of these complexes is only intermediate and the bioavailability is reduced.

Human insulin has three primary amino groups: the N-terminal group of the A-chain and of the B-chain and the ϵ -amino group of Lys^{B29}. Several insulin derivatives which are substituted in one or more of these groups are known in the prior art. Thus, US Patent No. 3,528,960 (Eli Lilly) relates to N-carboxyaroyl insulins in which one, two or three primary amino groups of the

insulin molecule has a carboxyaroyl group. No specifically N^{εB29}-substituted insulins are disclosed.

According to GB Patent No. 1.492.997 (Nat. Res. Dev. Corp.), it has been found that insulin with a carbamyl substitution at N^{εB29} has an improved profile of hypoglycaemic effect.

JP laid-open patent application No. 1-254699 (Kodama Co., Ltd.) discloses insulin wherein a fatty acid is bound to the amino group of Phe^{B1} or to the ε-amino group of Lys^{B29} or to both of these. The stated purpose of the derivatisation is to obtain a pharmacologically acceptable, stable insulin preparation.

Insulins, which in the B30 position have an amino acid having at least five carbon atoms which cannot necessarily be coded for by a triplet of nucleotides, are described in JP laid-open patent application No. 57-067548 (Shionogi). The insulin analogues are claimed to be useful in the treatment of diabetes mellitus, particularly in patients who are insulin resistant due to generation of bovine or swine insulin antibodies.

By "insulin derivative" as used herein is meant a compound having a molecular structure similar to that of human insulin including the disulfide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulfide bridge between Cys^{A6} and Cys^{A11}, and which have insulin activity.

However, there still is a need for protracted injectable insulin compositions which are solutions and contain insulins which stay in solution after injection and possess minimal inflammatory and immunogenic properties.

One object of the present invention is to provide human insulin derivatives, with a protracted profile of action, which are soluble at physiological pH values.

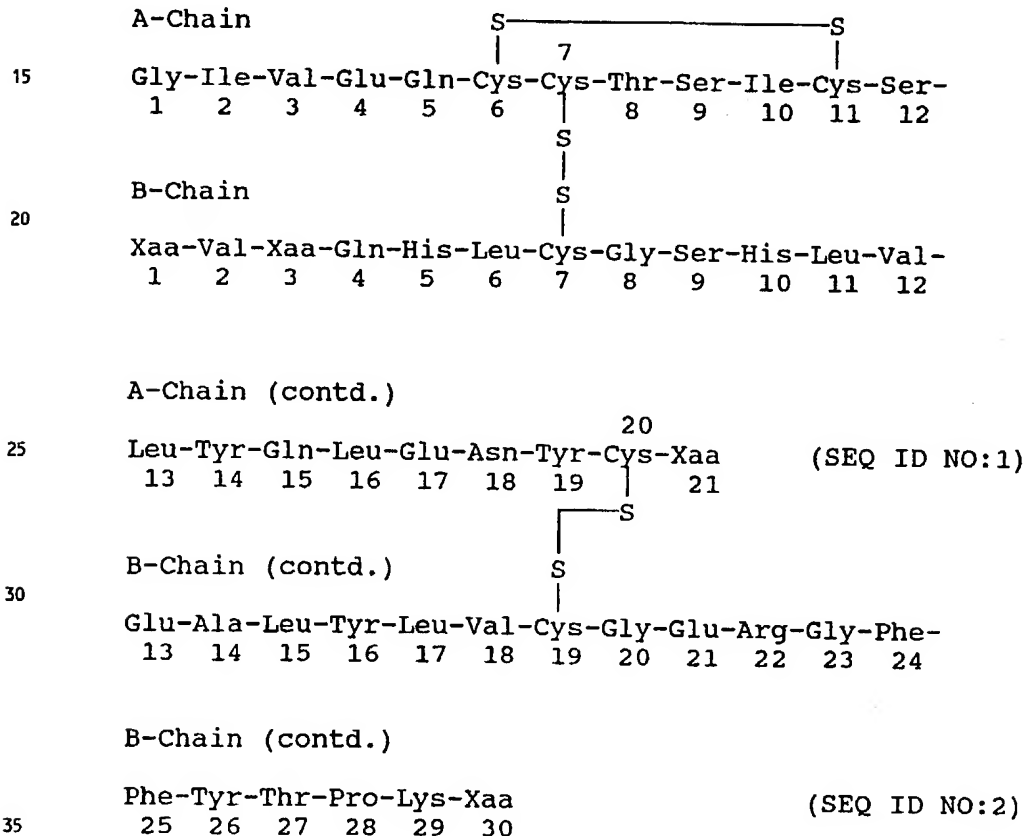
Another object of the present invention is to provide a pharmaceutical composition comprising the human insulin derivatives according to the invention.

It is a further object of the invention to provide a method of making the human insulin derivatives of the invention.

SUMMARY OF THE INVENTION

Surprisingly, it has turned out that certain human insulin derivatives, wherein the ϵ -amino group of Lys^{B29} has a lipophilic substituent, have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to an insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

5 Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ -amino group of Lys^{B29}, (b) any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent; and any Zn²⁺ complexes thereof, provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn²⁺ complex.

20 In one preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues
25 which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn²⁺ ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both
30 Asn, and Phe^{B1} is not deleted, then 2-4 Zn²⁺ ions are bound to each hexamer of the insulin derivative.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by
35 the genetic code except Lys, Arg and Cys; the A21 and the B3

amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys, with the proviso that if the B30 amino acid residue is Ala or Thr, then at least one of the residues A21 and B3 is different from Asn; Phe^{B1} may be deleted; and the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn²⁺ ions are bound to each insulin hexamer.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Glu.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic amino acid having at least 10 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic α -amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a straight chain, saturated, aliphatic α -amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is D- or L-N^ε-dodecanoyllysine.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino decanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino undecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino dodecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino tridecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino tetradecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino pentadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino hexadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is an α -amino acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ala.

10 In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gly.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ser.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Gln.

25 In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a carboxylic acid having at least 6 carbon atoms.

5 In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group, branched or unbranched, which corresponds to a carboxylic acid having a chain of carbon atoms 8 to 24 atoms long.

10 In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a fatty acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a
15 human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a
20 human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 8 to 12 carbon atoms.

In another preferred embodiment, the invention relates to a
25 human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 10 to 16 carbon atoms.

In another preferred embodiment, the invention relates to a
30 human insulin derivative in which the ϵ -amino group of Lys^{B29} has

a lipophilic substituent which is an oligo oxyethylene group comprising up to 10, preferably up to 5, oxyethylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has
5 a lipophilic substituent which is an oligo oxypropylene group comprising up to 10, preferably up to 5, oxypropylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 2 Zn²⁺ ions.

10 In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 3 Zn²⁺ ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 4
15 Zn²⁺ ions.

In another preferred embodiment, the invention relates to the use of a human insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment, the invention relates to a
20 pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

25 In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention, in mixture with an insulin or an

insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at physiological pH values.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at pH values in the interval from about 6.5 to about 8.5.

In another preferred embodiment, the invention relates to a protracted pharmaceutical composition comprising a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a pharmaceutical composition which is a solution containing from about 120 nmol/ml to about 1200 nmol/ml, preferably about 600 nmol/ml of a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

Examples of preferred human insulin derivatives according to the present invention in which no Zn^{2+} ions are bound are the following:

- N^εB29-tridecanoyl des(B30) human insulin,
- 5 N^εB29-tetradecanoyl des(B30) human insulin,
- N^εB29-decanoyl des(B30) human insulin,
- N^εB29-dodecanoyl des(B30) human insulin,
- N^εB29-tridecanoyl Gly^{A21} des(B30) human insulin,
- N^εB29-tetradecanoyl Gly^{A21} des(B30) human insulin,
- 10 N^εB29-decanoyl Gly^{A21} des(B30) human insulin,
- N^εB29-dodecanoyl Gly^{A21} des(B30) human insulin,
- N^εB29-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- N^εB29-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- 15 N^εB29-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- N^εB29-tridecanoyl Ala^{A21} des(B30) human insulin,
- N^εB29-tetradecanoyl Ala^{A21} des(B30) human insulin,
- N^εB29-decanoyl Ala^{A21} des(B30) human insulin,
- N^εB29-dodecanoyl Ala^{A21} des(B30) human insulin,
- 20 N^εB29-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^εB29-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^εB29-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^εB29-tridecanoyl Gln^{B3} des(B30) human insulin,
- 25 N^εB29-tetradecanoyl Gln^{B3} des(B30) human insulin,
- N^εB29-decanoyl Gln^{B3} des(B30) human insulin,
- N^εB29-dodecanoyl Gln^{B3} des(B30) human insulin,
- N^εB29-tridecanoyl Gly^{A21} human insulin,
- N^εB29-tetradecanoyl Gly^{A21} human insulin,
- 30 N^εB29-decanoyl Gly^{A21} human insulin,
- N^εB29-dodecanoyl Gly^{A21} human insulin,
- N^εB29-tridecanoyl Gly^{A21} Gln^{B3} human insulin,
- N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} human insulin,
- N^εB29-decanoyl Gly^{A21} Gln^{B3} human insulin,
- 35 N^εB29-dodecanoyl Gly^{A21} Gln^{B3} human insulin,
- N^εB29-tridecanoyl Ala^{A21} human insulin,

- $N^{\epsilon 829}$ -tetradecanoyl Ala^{A21} human insulin,
 $N^{\epsilon 829}$ -decanoyl Ala^{A21} human insulin,
 $N^{\epsilon 829}$ -dodecanoyl Ala^{A21} human insulin,
 $N^{\epsilon 829}$ -tridecanoyl Ala^{A21} Gln^{B3} human insulin,
5 $N^{\epsilon 829}$ -tetradecanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon 829}$ -decanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon 829}$ -dodecanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon 829}$ -tridecanoyl Gln^{B3} human insulin,
 $N^{\epsilon 829}$ -tetradecanoyl Gln^{B3} human insulin,
10 $N^{\epsilon 829}$ -decanoyl Gln^{B3} human insulin,
 $N^{\epsilon 829}$ -dodecanoyl Gln^{B3} human insulin,
 $N^{\epsilon 829}$ -tridecanoyl Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tetradecanoyl Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -decanoyl Glu^{B30} human insulin,
15 $N^{\epsilon 829}$ -dodecanoyl Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tridecanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tetradecanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -decanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -dodecanoyl Gly^{A21} Glu^{B30} human insulin,
20 $N^{\epsilon 829}$ -tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tridecanoyl Ala^{A21} Glu^{B30} human insulin,
25 $N^{\epsilon 829}$ -tetradecanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -decanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -dodecanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
30 $N^{\epsilon 829}$ -decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tridecanoyl Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -tetradecanoyl Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon 829}$ -decanoyl Gln^{B3} Glu^{B30} human insulin and
35 $N^{\epsilon 829}$ -dodecanoyl Gln^{B3} Glu^{B30} human insulin.

Examples of preferred human insulin derivatives according to the present invention in which two Zn^{2+} ions are bound per insulin hexamer are the following:

- (N^εB29-tridecanoyl des(B30) human insulin)₆, 2Zn²⁺,
- 5 (N^εB29-tetradecanoyl des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- 10 (N^εB29-decanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- 15 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- 20 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- 25 (N^εB29-tetradecanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl human insulin)₆, 2Zn²⁺,
- 30 (N^εB29-decanoyl human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- 35 (N^εB29-dodecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,

- (N^ε^{B29}-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-decanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-dodecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 5 (N^ε^{B29}-tetradecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-decanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-dodecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 10 (N^ε^{B29}-decanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-dodecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-decanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 15 (N^ε^{B29}-dodecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Gln^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-decanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-dodecanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
 20 (N^ε^{B29}-tridecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-decanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-dodecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 25 (N^ε^{B29}-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 30 (N^ε^{B29}-decanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-dodecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 35 (N^ε^{B29}-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tridecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,

(N^εB29-decanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺ and
 (N^εB29-dodecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺.

Examples of preferred human insulin derivatives according to the present invention in which three Zn²⁺ ions are bound per 5 insulin hexamer are the following:

- (N^εB29-tridecanoyl des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tetradecanoyl des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-decanoyl des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-dodecanoyl des(B30) human insulin)₆, 3Zn²⁺,
- 10 (N^εB29-tridecanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tetradecanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-decanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-dodecanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- 15 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tridecanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tetradecanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- 20 (N^εB29-decanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-dodecanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- 25 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tridecanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-tetradecanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-decanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB29-dodecanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- 30 (N^εB29-tridecanoyl human insulin)₆, 3Zn²⁺,
- (N^εB29-tetradecanoyl human insulin)₆, 3Zn²⁺,
- (N^εB29-decanoyl human insulin)₆, 3Zn²⁺,
- (N^εB29-dodecanoyl human insulin)₆, 3Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,
- 35 (N^εB29-tetradecanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,

- (N^εB29-decanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 5 (N^εB29-decanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 10 (N^εB29-dodecanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 15 (N^εB29-tridecanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 20 (N^εB29-tetradecanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 25 (N^εB29-decanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 30 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 35 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,

- (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺ and
 5 (N^εB29-dodecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺.

Examples of preferred human insulin derivatives according to the present invention in which four Zn²⁺ ions are bound per insulin hexamer are the following:

- (N^εB29-tridecanoyl des(B30) human insulin)₆, 4Zn²⁺,
 10 (N^εB29-tetradecanoyl des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 15 (N^εB29-decanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 20 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 25 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 30 (N^εB29-tetradecanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl human insulin)₆, 4Zn²⁺,
 35 (N^εB29-decanoyl human insulin)₆, 4Zn²⁺,

- (N^εB29-dodecanoyl human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 5 (N^εB29-dodecanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 10 (N^εB29-tridecanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 15 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gln^{B3} human insulin)₆, 4Zn²⁺,
 20 (N^εB29-decanoyl Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 25 (N^εB29-dodecanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 30 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 35 (N^εB29-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,

(N^ε^{B29}-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^ε^{B29}-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^ε^{B29}-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 5 (N^ε^{B29}-tridecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^ε^{B29}-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^ε^{B29}-decanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺ and
 (N^ε^{B29}-dodecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺.

BRIEF DESCRIPTION OF THE DRAWINGS

10 The present invention is further illustrated with reference to the appended drawings wherein

Fig. 1 shows the construction of the plasmid pEA5.3.2;

Fig. 2 shows the construction of the plasmid pEA108; and

Fig. 3 shows the construction of the plasmid pEA113.

15 DETAILED DESCRIPTION OF THE INVENTION

Terminology

The three letter codes and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. 243, p. 3558 (1968).

20 In the DNA sequences, A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms are used:

DMSO for dimethyl sulphoxide, DMF for dimethylformamide, Boc for tert-butoxycarbonyl, RP-HPLC for reversed phase high
 25 performance liquid chromatography, X-OSu is an N-hydroxysuccinimid ester, X is an acyl group, and TFA for trifluoroacetic acid.

Preparation of lipophilic insulin derivatives

The insulin derivatives according to the present invention can be prepared i.a. as described in the following:

1. Insulin derivatives featuring in position B30 an amino acid residue which can be coded for by the genetic code, e.g. threonine (human insulin) or alanine (porcine insulin).

1.1 Starting from human insulin.

Human insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc human insulin, i.e., human insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ -amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, N ^{ϵ B29}-X human insulin, is isolated.

1.2 Starting from a single chain insulin precursor.

A single chain insulin precursor, extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and in which the bridge from B30 to A1 is an arginine residue, i.e. a compound of the general formula Ext-Arg-B(1-30)-Arg-A(1-21), can be used as starting material. Acylation of this starting material with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ -amino group of Lys^{B29} and in the N-terminal amino group of the precursor. On treating this acylated precursor of the formula (N ^{ϵ B29}-X),X-Ext-Arg-B(1-30)-

Arg-A(1-21) with trypsin in a mixture of water and a suitable water-miscible organic solvent, e.g. DMF, DMSO or a lower alcohol, an intermediate of the formula $(N^{\epsilon B29}-X), Arg^{B31}$ insulin is obtained. Treating this intermediate with carboxypeptidase 5 B yields the desired product, $(N^{\epsilon B29}-X)$ insulin.

2. Insulin derivatives with no amino acid residue in position B30, i.e. des(B30) insulins.

2.1 Starting from human insulin or porcine insulin.

On treatment with carboxypeptidase A in ammonium buffer, human 10 insulin and porcine insulin both yield des(B30) insulin. After an optional purification, the des(B30) insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc des(B30) insulin, i.e., des(B30) insulin in which the N-terminal end of both chains are protected by a Boc-group. After 15 an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ -amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, 20 $(N^{\epsilon B29}-X)$ des(B30) insulin, is isolated.

2.2 Starting from a single chain human insulin precursor.

A single chain human insulin precursor, which is extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and which has a bridge from B30 to A1 25 can be a useful starting material. Preferably, the bridge is a peptide of the formula Y_n -Arg, where Y is a codable amino acid except lysine and arginine, and n is zero or an integer between 1 and 35. When $n > 1$, the Y's may designate different amino acids. Preferred examples of the bridge from B30 to A1 are: 30 AlaAlaArg, SerArg, SerAspAspAlaArg and Arg (European Patent No.

163529). Treatment of such a precursor of the general formula Ext-Arg-B(1-30)-Y_n-Arg-A(1-21) with a lysyl endopeptidase, e.g. Achromobacter lyticus protease, yields Ext-Arg-B(1-29) Thr-Y_n-Arg-A(1-21) des(B30) insulin. Acylation of this intermediate
 5 with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ε-amino group of Lys⁸²⁹, and in the N-terminal amino group of the A-chain and the B-chain to give (N^{ε829}-X) X-Ext-Arg-B(1-29) X-Thr-Y_n-Arg-A(1-21) des(B30) insulin. This intermediate on
 10 treatment with trypsin in mixture of water and a suitable organic solvent, e.g. DMF, DMSO or a lower alcohol, gives the desired derivative, (N^{ε829}-X) des(B30) human insulin.

Data on N^{ε829} modified insulins.

Certain experimental data on N^{ε829} modified insulins are given in
 15 Table 1.

The lipophilicity of an insulin derivative relative to human insulin, k'_{rel} , was measured on a LiChrosorb RP18 (5μm, 250x4 mm) HPLC column by isocratic elution at 40°C using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10%
 20 acetonitrile, and B) 50% acetonitrile in water as eluents. The elution was monitored by following the UV absorption of the eluate at 214 nm. Void time, t_0 , was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, t_{human} , was adjusted to at least $2t_0$ by varying the ratio between the A and
 25 B solutions. $k'_{rel} = (t_{derivative} - t_0) / (t_{human} - t_0)$.

The degree of prolongation of the blood glucose lowering effect was studied in rabbits. Each insulin derivative was tested by subcutaneous injection of 12 nmol thereof in each of six rabbits in the single day retardation test. Blood sampling for
 30 glucose analysis was performed before injection and at 1, 2, 4 and 6 hours after injection. The glucose values found are expressed as percent of initial values. The Index of

Protraction, which was calculated from the blood glucose values, is the scaled Index of Protraction (prolongation), see p. 211 in Markussen et al., Protein Engineering 1 (1987) 205-213. The formula has been scaled to render a value of 100 with 5 bovine ultralente insulin and a value of 0 with Actrapid® insulin (Novo Nordisk A/S, 2880 Bagsvaerd, Denmark).

The insulin derivatives listed in Table 1 were administered in solutions containing 3 Zn^{2+} per insulin hexamer, except those specifically indicated to be Zn-free.

10 For the very protracted analogues the rabbit model is inadequate because the decrease in blood glucose from initial is too small to estimate the index of protraction. The prolongation of such analogues is better characterized by the disappearance rate in pigs. $T_{50\%}$ is the time when 50% of the 15 A14 Tyr(^{125}I) analogue has disappeared from the site of injection as measured with an external γ -counter (Ribbel, U et al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. serrano-Rios and P.J. Lefebvre (Eds): Diabetes 1985; Proceedings of the 12th Congress of the International Diabetes 20 Federation, Madrid, Spain, 1985 (Excerpta Medica, Amsterdam, (1986) 891-96).

In Table 2 are given the $T_{50\%}$ values of a series of very protracted insulin analogues. The analogues were administered in solutions containing 3 Zn^{2+} per insulin hexamer.

Table 1

Insulin Derivative *)	Relative Lipophilicity	Blood glucose, % of initial				Index of protraction
		1h	2h	4h	6h	
N ^ε 829-benzoyl insulin	1.14					
N ^ε 829-phenylacetyl insulin (Zn-free)	1.28	55.4	58.9	88.8	90.1	10
N ^ε 829-cyclohexylacetyl insulin	1.90	53.1	49.6	66.9	81.1	28
N ^ε 829-cyclohexylpropionyl insulin	3.29	55.5	47.6	61.5	73.0	39
N ^ε 829-cyclohexylvaleroyl insulin	9.87	65.0	58.3	65.7	71.0	49
N ^ε 829-octanoyl insulin	3.97	57.1	54.8	69.0	78.9	33
N ^ε 829-decanoyl, des(B30) insulin	11.0	74.3	65.0	60.9	64.1	65
N ^ε 829-decanoyl insulin	12.3	73.3	59.4	64.9	68.0	60
N ^ε 829-undecanoyl, des(B30) insulin	19.7	88.1	80.0	72.1	72.1	80
N ^ε 829-lauroyl, des(B30) insulin	37.0	91.4	90.0	84.2	83.9	78
N ^ε 829-myristoyl insulin	113	98.5	92.0	83.9	84.5	97
N ^ε 829-choloyl insulin	7.64	58.2	53.2	69.0	88.5	20
N ^ε 829-7-deoxycholoyl insulin (Zn-free)	24.4	76.5	65.2	77.4	87.4	35
N ^ε 829-lithocholoyl insulin (Zn-free)	51.6	98.3	92.3	100.5	93.4	115
N ^ε 829-4-benzoyl-phenylalanyl insulin	2.51	53.9	58.7	74.4	89.0	14
N ^ε 829-3,5-diiodotyrosyl insulin	1.07	53.9	48.3	60.8	82.1	27
N ^ε 829-L-thyroxyl insulin	8.00					

Table 2

Derivative of Human Insulin	Relative hydrophobicity	Subcutaneous disappearance in pigs
5 600 μ M, 3Zn ²⁺ /hexamer, phenol 0.3%, glycerol 1.6%, pH 7.5	k'_{rel}	$T_{50\%}$, hours
10 N ^ε B ²⁹ decanoyl des(B30) insulin	11.0	5.6
N ^ε B ²⁹ undecanoyl des(B30) insulin	19.7	6.9
N ^ε B ²⁹ lauroyl des(B30) insulin	37	10.1
15 N ^ε B ²⁹ tridecanoyl des(B30) insulin	65	12.9
N ^ε B ²⁹ myristoyl des(B30) insulin	113	13.8
20 N ^ε B ²⁹ palmitoyl des(B30) insulin	346	12.4
N ^ε B ²⁹ succinimido- myristic acid insulin	10.5	13.6
25 N ^ε B ²⁹ myristoyl insulin	113	11.9
Human NPH		10

Solubility

The solubility of all the N^εB²⁹ modified insulins mentioned in Table 1, which contain 3 Zn²⁺ ions per insulin hexamer, exceeds 30 600 nmol/ml in a neutral (pH 7.5), aqueous, pharmaceutical formulation which further comprises 0.3% phenol as preservative, and 1.6% glycerol to achieve isotonicity. 600 nmol/ml is the concentration of human insulin found in the 100 IU/ml compositions usually employed in the clinic.

The ϵ -B29 amino group can be a component of an amide bond, a sulphonamide bond, a carbamide, a thiocarbamide, or a carbamate. The lipophilic substituent carried by the ϵ -B29 amino group can also be an alkyl group.

5 Pharmaceutical compositions containing a human insulin derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means
10 of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the human insulin derivative in the form of a nasal spray.

15 The injectable human insulin compositions of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

Thus, according to one procedure, the human insulin derivative
20 is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium
25 hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride, mannitol and glycerol.

30 Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

A composition for nasal administration of an insulin derivative according to the present invention may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific human insulin derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Where expedient, the human insulin derivatives of this invention may be used in mixture with other types of insulin, e.g. human insulin or porcine insulin or insulin analogues with a more rapid onset of action. Examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826 (Novo Nordisk A/S), EP 375437 (Novo Nordisk A/S) and EP 383472 (Eli Lilly & Co.).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Plasmids and DNA material

All expression plasmids are of the cPOT type. Such plasmids are described in EP patent application No. 171 142 and are characterized in containing the Schizosaccharomyces pombe triose phosphate isomerase gene (POT) for the purpose of plasmid selection and stabilization. A plasmid containing the POT-gene is available from a deposited E. coli strain (ATCC 39685). The plasmids furthermore contain the S. cerevisiae triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pMT742 (Egel-Mitani, M. et al., Gene 73 (1988) 113-120) (see Fig. 1) except for the region defined by the ECoRI-XbaI restriction sites encompassing the coding region for signal/leader/product.

Synthetic DNA fragments were synthesized on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S.L. and Caruthers, M.H., Tetrahedron Letters 22 (1981) 1859-1869).

All other methods and materials used are common state of the art knowledge (see, e.g. Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989).

Analytical

Molecular masses of the insulins prepared were obtained by MS (mass spectroscopy), either by PDMS (plasma desorption mass spectrometry) using a Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden) or by ESMS (electrospray mass spectrometry) using an API III Biomolecular Mass Analyzer (Perkin-Elmer Sciex Instruments, Thornhill, Canada).

EXAMPLE 1

Synthesis of Ala^{A21} Asp^{B3} human insulin precursor from Yeast strain yEA002 using the Lac212spx3 signal/leader.

5 The following oligonucleotides were synthesized:

- #98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCA
CTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAA
AGAGGTTTCTTCTACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3})
(SEQ ID NO:3)
- 10 #128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTC
GTCAGACTTTGG-3' (Ala^{A21}) (SEQ ID NO:4)
- #126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3})
(SEQ ID NO:5)
- 15 #16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Ave., CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with
20 100 µl of mineral oil (Sigma Chemical Co., St. Louis, MO, USA).

- 2.5 µl of oligonucleotide #98 (2.5 pmol)
2.5 µl of oligonucleotide #128 (2.5 pmol)
10 µl of 10X PCR buffer
16 µl of dNTP mix
25 0.5 µl of Taq enzyme
58.5 µl of water

One cycle was performed: 94°C for 45 sec., 49°C for 1 min, 72°C for 2 min.

Subsequently, 5µl of oligonucleotides #16 and #126 was added
30 and 15 cycles were performed: 94°C for 45 sec., 45°C for 1 min, 72°C for 1.5 min. The PCR mixture was loaded onto a 2.5 %

agarose gel and subjected to electrophoresis using standard techniques (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989). The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean Kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacturer's instructions. The purified PCR DNA fragment was dissolved in 10 μ l of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and Xba I according to standard techniques, run on a 2.5% agarose gel and purified using the Gene Clean Kit as described.

The plasmid pAK188 consists of a DNA sequence of 412 bp composed of a EcoRI/NcoI fragment encoding the synthetic yeast signal/leader gene LaC212spx3 (described in Example 3 of WO 89/02463) followed by a synthetic NcoI/XbaI fragment encoding the insulin precursor MI5, which has a SerAspAspAlaLys bridge connecting the B29 and the A1 amino acid residues (see SEQ ID NOS. 14, 15 and 16), inserted into the EcoRI/XbaI fragment of the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK188 is shown in Fig. 1.

The plasmid pAK188 was also cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3139 bp isolated. The two DNA fragments were ligated together using T4 DNA ligase and standard conditions (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989). The ligation mixture was transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using standard DNA miniprep technique (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989), checked with appropriate restrictions endonucleases i.e. EcoRI, Xba I, NcoI and HpaI. The selected plasmid was shown by DNA sequencing analyses (Sequenase, U.S. Biochemical Corp.) to contain the correct sequence for the Ala^{A21}, Asp^{B3} human insulin precursor and named pEA5.3.

The plasmid pKFN1627 is an E. coli - S. cerevisiae shuttle vector, identical to plasmid pKFN1003 described in EP patent No. 375718, except for a short DNA sequence upstream from the unique XbaI site. In pKFN1003, this sequence is a 178 bp
5 fragment encoding a synthetic aprotinin gene fused in-frame to the yeast mating factor alpha 1 signal-leader sequence. In pKFN1627, the corresponding 184 bp sequence encodes the insulin precursor MI5 (Glu^{B1}, Glu^{B28}) (i.e. B(1-29, Glu^{B1}, Glu^{B28})-SerAspAspAlaLys-A(1-21) fused in-frame to the mating factor
10 alpha 1 sequence (see SEQ ID NOS. 17, 18 and 19). The vector pKFN1627 is shown in Fig. 1.

pEA5.3 was cut with the restriction endonucleases EcoRI and XbaI and the resulting DNA fragment of 412 bp was isolated. The yeast expression vector pKFN1627 was cut with the restriction
15 endonucleases NcoI and XbaI and with NcoI and EcoRI and the DNA fragment of 9273 bp was isolated from the first digestion and the DNA fragment of 1644 bp was isolated from the second. The 412 bp EcoRI/XbaI fragment was then ligated to the two other fragments, that is the 9273 bp NcoI I/XbaI fragment and the
20 1644 bp NcoI/EcoRI fragment using standard techniques.

The ligation mixture was transformed into E. coli as described above. Plasmid from the resulting E. coli was isolated using standard techniques, and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, Hpa I. The selected
25 plasmid was shown by DNA sequence analysis (using the Sequenase kit as described by the manufacturer, U.S. Biochemical) to contain the correct sequence for the Ala^{A21} Asp^{B3} human insulin precursor DNA and to be inserted after the DNA encoding the LaC212spx3 signal/leader DNA. The plasmid was named pEA5.3.2
30 and is shown in Fig. 1. The DNA sequence encoding the LaC212spx3 signal/leader/Ala^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 20, 21 and 22. The plasmid pEA5.3.2 was transformed into S. cerevisiae strain MT663 as described in European patent

application having the publication No. 214826 and the resulting strain was named yEA002.

EXAMPLE 2

Synthesis of Ala^{A21} Thr^{B3} human insulin precursor from Yeast strain yEA005 using the Lac212spx3 signal/leader.

The following oligonucleotides were synthesized:

- #101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT
GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA
10 CTCCAAAGTCTGACGACGCT-3' (Thr^{B3}) (SEQ ID NO:7)
#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAAA
GAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCG
TCAGACTTTGG-3' (Ala^{A21}) (SEQ ID NO:4)
#15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (Thr^{B3}) (SEQ ID
15 NO:8)
#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Ala^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The
20 DNA sequence encoding the Lac212spx3 signal/leader/Ala^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 23, 24 and 25. The plasmid pEA8.1.1 was shown to contain the desired sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the
25 resulting strain was named yEA005.

EXAMPLE 3

Synthesis of Gly^{A21} Asp^{B3} human insulin precursor from Yeast strain yEA007 using the Lac212spx3 signal/leader.

30 The following oligonucleotides were synthesized:

- #98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCACTTG
GTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCT
ACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3}) (SEQ ID NO:3)
- #127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
5 AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTCGTCAGACTTTGG-3' (Gly^{A21}) (SEQ ID NO:9)
- #126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3}) (SEQ ID
NO:5)
- #16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)
- 10 The DNA encoding Gly^{A21} Asp^{B3} human insulin precursor was
constructed in the same manner as described for the DNA
encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The
DNA sequence encoding the Lac212spx3 signal/leader/Gly^{A21} Asp^{B3}
human insulin precursor complex and the amino acid sequence
15 thereof are SEQ ID NOS. 26, 27 and 28. The plasmid pEA1.5.6 was
shown to contain the desired sequence, transformed into *S.*
cerevisiae strain MT663 as described in Example 1 and the
resulting strain was named yEA007.

EXAMPLE 4

- 20 Synthesis of Gly^{A21} Thr^{B3} human insulin precursor from Yeast
strain yEA006 using the Lac212spx3 signal/leader.

The following oligonucleotides were synthesized:

- #101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT
25 GGTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA
CTCCAAAGTCTGACGACGCT-3' (Thr^{B3}) (SEQ ID NO:7)
- #127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTCGTCAGACTTTGG-3' (Gly^{A21}) (SEQ ID NO:9)
- 30 #15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (Thr^{B3}) (SEQ ID
NO:8)
- #16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Gly^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Gly^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 29, 30 and 31. The plasmid pEA4.4.11 was shown to contain the desired DNA sequence, transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA006.

10 EXAMPLE 5

Synthesis of Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) from Yeast strain yEA113 using the alpha factor leader.

15 A)

The following oligonucleotides were synthesized:

- #220 5'-ACGTACGTTCTAGAGCCTGCGGGCTGC-3' (SEQ ID NO:10)
 #263 5'-CACTTGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTC
 TTCTACACTCCAAAGACTAGAGGTATCGTTGAA-3' (SEQ ID NO:11)
 20 #307 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCTGAAGCT
 AGATTTCGTTAACCAACAC-3' (SEQ ID NO:12)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Ave., Walk, CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with 100 µl of mineral oil (Sigma Chemical Co, St. Louis, MO, USA). The plasmid pAK220 (which is identical to pAK188) consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the insulin precursor MI5 (see SEQ ID NOS. 14, 15 and 16) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA).

5 μ l of oligonucleotide #220 (100 pmol)
5 μ l of oligonucleotide #263 (100 pmol)
10 μ l of 10X PCR buffer
16 μ l of dNTP mix
5 0.5 μ l of Taq enzyme
0.5 μ l of pAK220 plasmid (identical to pAK188) as template (0.2
 μ g of DNA)
63 μ l of water

A total of 16 cycles were performed, each cycle comprising 1
10 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The
PCR mixture was then loaded onto a 2% agarose gel and subjected
to electrophoresis using standard techniques. The resulting DNA
fragment was cut out of the agarose gel and isolated using the
Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038,
15 USA) according to the manufacture's instructions. The purified
PCR DNA fragment was dissolved in 10 μ l of water and
restriction endonuclease buffer and cut with the restriction
endonucleases HindIII and XbaI according to standard
techniques. The HindIII/XbaI DNA fragment was purified using
20 The Gene Clean Kit as described.

The plasmid pAK406 consists of a DNA sequence of 520 bp
comprising an EcoRI/HindIII fragment derived from pMT636
(described in WO 90/10075) encoding the yeast alpha factor
leader and part of the insulin precursor ligated to the
25 HindIII/XbaI fragment from pAK188 encoding the rest of the
insulin precursor MI5 (see SEQ ID NOS. 32, 33 and 34) inserted
into the vector cPOT. The vector pAK406 is shown in Fig. 2.

The plasmid pAK233 consists of a DNA sequence of 412 bp
encoding the synthetic yeast signal/leader Lac212sp_{x3}
30 (described in Example 3 of WO 89/02463) followed by the gene
for the insulin precursor B(1-29)-GluLysArg-A(1-21) (A21-Gly)
(see SEQ ID NOS. 35, 36 and 37) inserted into the vector cPOT.
The plasmid pAK233 is shown in Fig. 2.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 9273 bp isolated. The plasmid pAK406 was cut with the restriction endonucleases NcoI and HindIII and the vector fragment of 2012 bp isolated. These two DNA fragments were ligated together with the HindIII/XbaI PCR fragment using T4 DNA ligase and standard conditions. The ligation mixture was then transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA and to be inserted after the DNA encoding the S. cerevisiae alpha factor DNA. The plasmid was named pEA108 and is shown in Fig. 2. The DNA sequence encoding the alpha factor leader/Arg^{B31} single chain human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 38, 39 and 40. The plasmid pEA 108 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA108.

B)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Ave., CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with 100 µl of mineral oil (Sigma Chemical Co., St. Louis, MO, USA)

5 µl of oligonucleotide #220 (100 pmol)
5 µl of oligonucleotide #307 (100 pmol)
10 µl of 10X PCR buffer
16 µl of dNTP mix
0.5 µl of Taq enzyme
0.2 µl of pEA108 plasmid as template (0.1 ug DNA)
63 µl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The PCR mixture was then loaded onto an 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacture's instructions. The purified PCR DNA fragment was dissolved in 10 µl of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques. The NcoI/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK401 consists of a DNA sequence of 523 bp composed of an EcoRI/NcoI fragment derived from pMT636 (described in WO 90/10075) (constructed by by introducing a NcoI site in the 3'-end of the alpha leader by site directed mutagenesis) encoding the alpha factor leader followed by a NcoI/XbaI fragment from pAK188 encoding the insulin precursor MI5 (see SEQ ID NOS. 41, 42 and 43) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK401 is shown in Fig. 3.

The plasmid pAK401 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3254 bp isolated and ligated together with the NcoI/XbaI PCR fragment. The ligation mixture was then transformed into a competent E. coli strain and plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI. The selected plasmid, named p113A (shown in Fig. 3), was cut with EcoRI and XbaI and the fragment of 535 bp isolated.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI, and with EcoRI/NcoI and the fragments of 9273 and 1644 bp isolated. These two DNA fragments were ligated together with the EcoRI/XbaI fragment from p113A using T4 DNA

ligase and standard conditions. The ligation mixture was then transformed into a competent *E. coli* strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA with the N-terminal extension
10 GluGluAlaGluAlaGluAlaArg and to be inserted after the DNA encoding the *S. cerevisiae* alpha factor DNA. The plasmid was named pEA113 and is shown in Fig. 3. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension
15 (GluGluAlaGluAlaGluAlaArg) and the amino acid sequence thereof are SEQ ID NOS. 44, 45 and 46. The plasmid pEA113 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA113.

EXAMPLE 6

20 Synthesis of Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) from Yeast strain yEA136 using the alpha factor leader.

The following oligonucleotide was synthesized:

25 #389 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCGAAG
CTGAAAGATTCGTTAACCAACAC-3' (SEQ ID NO:13)

The following PCR was performed using the Gene Amp PCR reagent kit

5 µl of oligonucleotide #220 (100 pmol)
30 5 µl of oligonucleotide #389 (100 pmol)
10 µl of 10X PCR buffer

16 μ l of dNTP mix
0.5 μ l of Taq enzyme
2 μ l of pEA113 plasmid as template (0.5 ug DNA)
63 μ l of water

5 A total of 12 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 37°C; and 2 minutes at 72°C.

The DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) was constructed in the same
10 manner as described for the DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) in Example 5. The plasmid was named pEA136. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain
15 human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) and the amino acid sequence thereof are SEQ ID NOS. 47, 48 and 49. The plasmid pEA136 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA136.

20 EXAMPLE 7

Synthesis of (A1,B1)-diBoc human insulin.

5 g of zinc-free human insulin was dissolved in 41.3 ml of DMSO. To the solution was added 3.090 ml of acetic acid. The
25 reaction was conducted at room temperature and initiated by addition of 565 mg of di-tert-butyl pyrocarbonate dissolved in 5.650 ml of DMSO. The reaction was allowed to proceed for 5½ hour and then stopped by addition of 250 μ l of ethanolamine. The product was precipitated by addition of 1500 ml of acetone.
30 The precipitate was isolated by centrifugation and dried in vacuum. A yield of 6.85 g material was obtained.

(A1,B1)-diBoc insulin was purified by reversed phase HPLC as follows: The crude product was dissolved in 100 ml of 25% ethanol in water, adjusted to pH 3.0 with HCl and applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μ m, pore size 100 Å) and equilibrated with elution buffer. The elution was performed using mixtures of ethanol and 1 mM aqueous HCl, 0.3 M KCl at a flow of 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 45%. The appropriate fraction was diluted to 20% ethanol and precipitated at pH 4.8. The precipitated material was isolated by centrifugation and dried in vacuum. Thus 1.701 g of (A1,B1)-diBoc human insulin was obtained at a purity of 94.5%.

EXAMPLE 8

15 Synthesis of ($N^{\epsilon 829}$ -benzoyl human insulin)₆, 3Zn²⁺.

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μ l of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 14.6 mg of benzoic acid N-hydroxysuccinimide ester dissolved in 132 μ l DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 343 mg of material was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum.

30 $N^{\epsilon 829}$ -benzoyl human insulin was purified by reversed phase HPLC as described in Example 7. A yield of 230 mg was obtained. Recrystallization from 15% aqueous ethanol containing 6 mM Zn²⁺

and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 190 mg.

Molecular mass, found by MS: 5911, theory: 5911.

5 EXAMPLE 9

Synthesis of (N^εB29-lithocholoyl human insulin)₆, 3Zn²⁺.

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μl of a mixture of N-
10 methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 31.94 mg of lithocholic acid N-hydroxysuccinimide ester dissolved in 300 μl of DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by
15 centrifugation and dried in vacuum. 331 mg of material was obtained.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The
20 precipitate was isolated by centrifugation and dried in vacuum. The yield was 376 mg.

B29-lithocholoyl insulin was purified by reversed phase HPLC as described in Example 7. A final yield of 67 mg was obtained at a purity of 94%. Recrystallization from 15% aqueous ethanol
25 containing 6 mM Zn²⁺ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 49 mg.

Molecular mass, found by MS: 6160, theory: 6166.

EXAMPLE 10

Synthesis of (N^ε⁸²⁹-decanoyl human insulin)₆, 3Zn²⁺.

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of
5 DMSO. To the solution was added 748 μl of a mixture of N-
methylemorpholine and DMSO (1:9, v/v). The reaction was
conducted at 15°C and initiated by addition of 18.0 mg of
decanoic acid N-hydroxysuccinimide ester dissolved in 132 μl of
DMF. The reaction was stopped after 60 minutes and the product
10 precipitated by addition of 100 ml of acetone. The precipitated
material was isolated by centrifugation and dried in vacuum.
420 mg of intermediate product was collected.

The Boc protecting groups were eliminated by addition of 4 ml
of TFA. The dissolved material was incubated for 30 minutes and
15 the product was then precipitated by addition of 50 ml of
acetone. The precipitate was isolated by centrifugation and
dried in vacuum. The yield of crude product was 420 mg.

The crude product was purified by reversed phase HPLC as
described in Example 7. A final yield of 254 mg of the title
20 product was obtained. The purity was 96.1%. Recrystallization
from 15% aqueous ethanol containing 6 mM Zn²⁺ and 50 mM citrate
at pH 5.5 gave crystals of the title compound which were
isolated by centrifugation and dried in vacuum. The yield was
217 mg.

25 Molecular mass, found by MS: 5962, theory: 5962.

EXAMPLE 11

Synthesis of des(B30) human insulin.

Synthesis of des(B30) human insulin was carried out as
30 described by Markussen (Methods in diabetes research, Vol. I,

Laboratory methods, part B, 404-410. Ed: J. Larner and S. Phol, John Wiley & Sons, 1984). 5 g of human insulin was dissolved in 500 ml of water while the pH value of the solution was kept at 2.6 by addition of 0.5 M sulphuric acid. Subsequently, the insulin was salted out by addition of 100 g of ammonium sulphate and the precipitate was isolated by centrifugation. The pellet was dissolved in 800 ml of 0.1 M ammonium hydrogen carbonate and the pH value of the solution was adjusted to 8.4 with 1 M ammonia.

50 mg of bovine carboxypeptidase A was suspended in 25 ml of water and isolated by centrifugation. The crystals were suspended in 25 ml of water and 1 M ammonia was added until a clear solution was obtained at a final pH of 10. The carboxypeptidase solution was added to the insulin solution and the reaction was allowed to proceed for 24 hours. A few drops of toluene were added to act as preservative during the reaction.

After 24 hours the des(B30) human insulin was crystallized by successive addition of 80 g of sodium chloride while the solution was stirred. The pH value was then adjusted to 8.3 and the crystallization was allowed to proceed for 20 hours with gentle stirring. The crystals were isolated on a 1.2 μ m filter, washed with 250 ml of ice cold 2-propanol and finally dried in vacuum.

EXAMPLE 12

Synthesis of (A1,B1)-diBoc des(B30) human insulin.

The title compound was synthesized by a method similar to that described in Example 7, using des(B30) porcine insulin as the starting material. The crude product was precipitated by acetone and dried in vacuum. The (A1,B1)-diBoc des(B30) human

insulin was purified by reversed phase HPLC as described in Example 7.

EXAMPLE 13

Synthesis of N^ε⁸²⁹-decanoyl des(B30) human insulin.

5

400 mg of (A1,B1)-diBoc des(B30) human insulin was used as starting material for the synthesis of N^ε⁸²⁹-decanoyl des(B30) human insulin, following the procedure described in Example 10. The crude product was precipitated by acetone, dried in vacuum
10 and deprotected using TFA. The resulting product was precipitated by acetone and dried in vacuum. N^ε⁸²⁹-decanoyl des(B30) human insulin was then purified by reversed phase HPLC as described in Example 10.

Molecular mass, found by MS: 5856, theory: 5861.

15 EXAMPLE 14

Synthesis of N^ε⁸²⁹-dodecanoyl des(B30) human insulin.

a. Immobilization of A. lyticus protease

13 mg of A. lyticus protease, dissolved in 5 ml of aqueous 0.2
20 M NaHCO₃ buffer, pH 9.4, was mixed with 4 ml of settled MiniLeak[®] Medium gel, which had been washed with the same buffer (MiniLeak is a divinylsulfone activated Sepharose CL 6B, obtained from KemEnTec, Copenhagen). The gel was kept in suspension by gentle stirring for 24 hours at room temperature.
25 Then, the gel was isolated by filtration, washed with water, and suspended in 20 ml of 1 M ethanolamine buffer, pH 9.4, and kept in suspension for 24 hours at room temperature. Finally, the gel was washed with water followed by 0.1 M acetic acid and stored at 4°C. The enzyme activity in the filtrate was 13% of

that in the initial solution, indicating a yield in the immobilization reaction of about 87%.

b. Immobilization of porcine trypsin

Porcine trypsin was immobilized to MiniLeak® Low to a degree of substitution of 1 mg per ml of gel, using the conditions described above for immobilization of A. lyticus.

c. Synthesis of Glu(GluAla)₃Arg-B(1-29), ThrArg-A(1-21) insulin using immobilized A. lyticus protease

To 200 mg of Glu(GluAla)₃Arg-B(1-29)-ThrArg-A(1-21) single-chain human insulin precursor, dissolved in 20 ml of 0.1 M NaHCO₃ buffer, pH 9.0, was added 4 ml of the gel carrying the immobilized A. lyticus protease. After the gel had been kept in suspension in the reaction mixture for 6 hours at room temperature the hydrolysis was complete, rendering Glu(GluAla)₃-Arg-B(1-29), ThrArg-A(1-21) human insulin (the reaction was followed by reversed phase HPLC). After the hydrolysis, the gel was removed by filtration. To the filtrate was added 5 ml of ethanol and 15 µL of 1 M ZnCl₂ and the pH was adjusted to 5.0 using HCl. The precipitation of the product was completed on standing overnight at 4°C with gentle stirring. The product was isolated by centrifugation. After one washing with 1 ml of ice cold 20% ethanol and drying in vacuo the yield was 190 mg.

d. Synthesis of N^{αA1},N^{αB1},N^{εB29}-tridodecanoyl Glu(GluAla)₃Arg-B(1-29), Thr-Arg-A(1-21) human insulin using dodecanoic acid N-hydroxysuccinimide ester

190 mg (30 µmol) of Glu(GluAla)₃Arg-B(1-29), ThrArg-A(1-21) insulin was dissolved in 1 ml of DMSO and 1.05 ml of a 0.572 M solution of N,N-diisopropylethylamine in DMF. The solution was cooled to 15°C and 36 mg (120 µmol) of dodecanoic acid N-hydroxysuccinimide ester dissolved in 0.6 ml of DMSO was added.

The reaction was completed within 24 hours. The lipophilic title compound was not isolated.

e. Synthesis of N^ε⁸²⁹-dodecanoyl des(B30) insulin

The product from the previous step, d., contained in approximately 2,65 ml of DMSO/DMF/N,N-diisopropylethylamine was diluted with 10.6 ml of a 50 mM glycine buffer comprising 20% ethanol and the pH adjusted to 10 with NaOH. After standing for 1 hour at room temperature 1 ml of MiniLeak gel, carrying 1 mg of immobilized trypsin per ml of gel, was added. The reaction mixture was stirred gently for 48 hours at room temperature. In order to isolate the desired product, the reaction mixture was applied to a reversed phase HPLC column (5 cm in diameter, 30 cm high), packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μ m, pore size 100 Å). For the elution was used 20 mM Tris/HCl buffers, adjusted to pH 7.7 and comprising an increasing concentration of ethanol, from 40% to 44% (v/v), at a rate of 2000 ml/h. The major peak eluting at about 43-44% of ethanol contained the title compound. The fractions containing the major peak were pooled, water was added to reduce the ethanol concentration to 20% (v/v), and the pH was adjusted to 5.5. The solution was left overnight at -20°C, whereby the product precipitated. The precipitate was isolated by centrifugation at -8°C and dried in vacuo. The yield of the title compound was 90 mg.

Molecular mass, found by MS: 5892, theory: 5890.

EXAMPLE 15

Synthesis of N^ε⁸²⁹-(N-myristoyl- α -glutamyl) human insulin.

500 mg of (A1,B1)-diBoc human insulin was dissolved in 2.5 ml of DMSO and 428 μ l of ethyl diisopropylamine, diluted with 2.5 ml of DMSO/DMF 1/1 (v/v), was added. The temperature was

adjusted to 15°C and 85 mg of N-myristoyl-Glu(OBut) N-hydroxysuccinimide ester, dissolved in 2.5 ml of DMSO/DMF 1/1 (v/v), was added. After 30 min the reaction mixture was poured into 60 ml of water, the pH adjusted to 5 and the precipitate
5 isolated by centrifugation. The precipitate was dried in vacuo. The dried reaction mixture was dissolved in 25 ml of TFA, and the solution was left for 30 min at room temperature. The TFA was removed by evaporation in vacuo. The gelatinous residue was dissolved in 60 ml of water and the pH was adjusted to 11.2
10 using concentrated ammonia. The title compound was crystallized from this solution by adjustment of the pH to 8.5 using 6 N HCl. The product was isolated by centrifugation, washed once by 10 ml of water, and dried in vacuo. Yield 356 mg. Purity by HPLC 94%.

15 The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_{12}\text{CONHCH}(\text{CH}_2\text{CH}_2\text{COOH})\text{CO}-$.

Molecular mass, found by MS: 6146, theory: 6148.

EXAMPLE 16

20 Synthesis of N^{εB29}-undecanoyl des(B30) human insulin.

The title compound was synthesized analogously to N^{εB29}-dodecanoyl des(B30) human insulin as described in Example 14, by using undecanoic acid N-hydroxysuccinimide ester instead of
25 dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5876, theory: 5876.

EXAMPLE 17

Synthesis of N^{ε829}-tridecanoyl des(B30) human insulin.

The title compound was synthesized analogously to N^{ε829}-
5 dodecanoyl des(B30) human insulin as described in Example 14,
by using tridecanoic acid N-hydroxysuccinimide ester instead of
dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5899, theory: 5904.

EXAMPLE 18

10 Synthesis of N^{ε829}-myristoyl des(B30) human insulin.

The title compound was synthesized analogously to N^{ε829}-
dodecanoyl des(B30) human insulin as described in Example 14,
by using myristic acid N-hydroxysuccinimide ester instead of
15 dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5923, theory: 5918.

EXAMPLE 19

Synthesis of N^{ε829}-palmitoyl des(B30) human insulin.

20 The title compound was synthesized analogously to N^{ε829}-
dodecanoyl des(B30) human insulin as described in Example 14,
by using palmitic acid N-hydroxysuccinimide ester instead of
dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5944, theory: 5946.

EXAMPLE 20

Synthesis of N^{εB29}-suberoyl-D-thyroxine human insulin.

a. Preparation of N-(succinimidylsuberoyl)-D-thyroxine.

5 Disuccinimidyl suberate (1.0 g, Pierce) was dissolved in DMF (50 ml), and D-thyroxine (2.0 g, Aldrich) was added with stirring at 20°C. The thyroxine slowly dissolved, and after 20 hours the solvent was removed by evaporation in vacuo. The oily residue was crystallized from 2-propanol to yield 0.6 g of N-
10 (succinimidylsuberoyl)-D-thyroxine, m.p. 128-133°C.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuberoyl)-D-thyroxine.

(A1,B1)-diBoc human insulin (200 mg) was dissolved in dry DMF (10 ml) by addition of triethylamine (20 μl) at room
15 temperature. Then, N-(succinimidylsuberoyl)-D-thyroxine (80 mg) was added. The reaction was monitored by reversed phase HPLC and when the reaction was about 90% complete, the solvent was removed in vacuo. To the evaporation residue, anhydrous trifluoroacetic acid (5 ml) was added, and the solution was
20 kept for 1 hour at room temperature. After removal of the trifluoroacetic acid in vacuo, the residue was dissolved in a mixture of 1M acetic acid (5 ml) and acetonitrile (1.5 ml), purified by preparative reversed phase HPLC and desalted on a PD-10 column. The yield of N^{εB29}-suberoyl-D-thyroxine human
25 insulin was 50 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: Thyrox-CO(CH₂)₆CO-, wherein Thyrox is thyroxine which is bound to the octanedioic acid moiety via an amide bond to
30 its α-amino group.

Molecular mass of the product found by MS: 6724, theory: 6723.

EXAMPLE 21

Synthesis of N^ε⁸²⁹-(2-succinylamido)myristic acid human insulin.

a. Preparation of α-aminomyristic acid methyl ester, HCl.

5 To methanol (5 ml, Merck) at -10°C, thionyl chloride (0.2 ml, Aldrich) was added dropwise while stirring vigorously. Then, α-aminomyristic acid (0.7 g, prepared from the α-bromo acid by reaction with ammonia) was added. The reaction mixture was stirred at room temperature overnight, and then evaporated to
10 dryness. The crude product (0.7 g) was used directly in step b.

b. Preparation of N-succinoyl-α-aminomyristic acid methyl ester.

α-Aminomyristic acid methyl ester, HCl (0.7 g) was dissolved in chloroform (25 ml, Merck). Triethylamine (0.35 ml, Fluka) was
15 added, followed by succinic anhydride (0.3 g, Fluka). The reaction mixture was stirred at room temperature for 2 hours, concentrated to dryness, and the residue recrystallized from ethyl acetate/petroleum ether (1/1). Yield: 0.8 g.

c. Preparation of N-(succinimidylsuccinoyl)-α-aminomyristic
20 acid methyl ester.

N-succinoyl-α-aminomyristic acid methyl ester (0.8 g) was dissolved in dry DMF (10 ml, Merck, dried over 4Å molecular sieve). Dry pyridine (80 μl, Merck), and di(N-succinimidyl)carbonate (1.8 g, Fluka) were added, and the reaction
25 mixture was stirred overnight at room temperature. The evaporation residue was purified by flash chromatography on silica gel 60 (Merck), and recrystallized from 2-propanol/petroleum ether (1/1). Yield of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester: 0.13
30 g, m.p. 64-66°C.

d. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester.

The reaction was carried out as in Example 20 b., but using N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester (16 mg) instead of N-(succinimidylsuberoyl)-D-thyroxine. After removal of the trifluoroacetic acid in vacuo, the evaporation residue was treated with 0.1M sodium hydroxide at 0°C to saponify the methyl ester. When the saponification was judged to be complete by reversed phase HPLC, the pH value in the solution was adjusted to 3, and the solution was lyophilized. After purification by preparative reversed phase HPLC and desalting on a PD-10 column, the yield of N^{εB29}-(2-succinylamido)myristic acid human insulin was 39 mg.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_{11}\text{CH}(\text{COOH})\text{NHCOCH}_2\text{CH}_2\text{CO}-$.

Molecular mass of the product found by MS: 6130, theory: 6133.

EXAMPLE 22

Synthesis of N^{εB29}-octyloxycarbonyl human insulin.

20

The synthesis was carried out as in Example 20 b., but using n-octyloxycarbonyl N-hydroxysuccinimide (9 mg, prepared from n-octyl chloroformate (Aldrich) and N-hydroxysuccinimide), instead of N-(succinimidylsuberoyl)-D-thyroxine. The yield of N^{εB29}-octyloxycarbonyl human insulin was 86 mg.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_7\text{OCO}-$.

Molecular mass of the product found by MS: 5960, theory: 5964.

EXAMPLE 23

Synthesis of N^{εB29}-(2-succinylamido)palmitic acid human insulin.

a. Preparation of N-(succinimidylsuccinoyl)-α-amino palmitic acid methyl ester.

This compound was prepared as described in Example 21 a.-c., using α-amino palmitic acid instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester.

10 The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester to give N^{εB29}-(2-succinylamido)palmitic acid human insulin.

15 The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: CH₃(CH₂)₁₃CH(COOH)NHCOCH₂CH₂CO-.

EXAMPLE 24

Synthesis of N^{εB29}-(2-succinylamidoethoxy)palmitic acid human
20 insulin.

a. Preparation of N-(succinimidylsuccinoyl)-2-aminoethoxy palmitic acid methyl ester.

This compound was prepared as described in Example 21 a.-c. but
25 using 2-aminoethoxy palmitic acid (synthesized by the general procedure described by R. TenBrink, J. Org. Chem. 52 (1987) 418-422 instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-2-aminoethoxypalmitic acid methyl ester.

The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-2-aminoethoxypalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester to give N^{ε829}-(2-succinylamidoethoxy)palmitic acid human insulin.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_{13}\text{CH}(\text{COOH})\text{NHCH}_2\text{CH}_2\text{OCOCH}_2\text{CH}_2\text{CO}-$.

EXAMPLE 25

Synthesis of N^{ε829}-lithocholoyl- α -glutamyl des(B30) human insulin.

15

The synthesis was carried out as in Example 13 using N-lithocholoyl-L-glutamic acid α -N-hydroxysuccinimide ester, γ -tert-butyl ester instead of decanoic acid N-hydroxysuccinimide ester.

20 The product of this example is thus des(B30) human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: lithocholoyl-NHCH(CH₂CH₂COOH)CO-.

Molecular mass of the product found by MS: 6194, theory: 6193.

EXAMPLE 26

Synthesis of N^{ε829}-3,3',5,5'-tetraiodothyroacetyl human insulin.

The synthesis was carried out as in Example 10 using 3,3',5,5'-
5 tetraiodothyroacetic acid N-hydroxysuccinimide ester, instead
of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6536, theory: 6538.

EXAMPLE 27

Synthesis of N^{ε829}-L-thyroxyl human insulin.

10

The synthesis was carried out as in Example 10 using Boc-L-
thyroxine N-hydroxysuccinimide ester, instead of decanoic acid
N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6572, theory: 6567.

15 EXAMPLE 28

A pharmaceutical composition comprising 600 nmol/ml of N^{ε829}-
decanoyl des(B30) human insulin, 1/3Zn²⁺ in solution.

N^{ε829}-decanoyl des(B30) human insulin (1.2 μmol) was dissolved in
20 water (0.8 ml) and the pH value was adjusted to 7.5 by addition
of 0.2 M sodium hydroxide. 0.01 M zinc acetate (60 μl) and a
solution containing 0.75% of phenol and 4% of glycerol (0.8 ml)
was added. The pH value of the solution was adjusted to 7.5
using 0.2 M sodium hydroxide and the volume of the solution was
25 adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and
transferred aseptically to a cartridge or a vial.

EXAMPLE 29

A pharmaceutical composition comprising 600 nmol/ml of N^ε⁸²⁹-decanoyl human insulin, $\frac{1}{2}$ Zn²⁺ in solution.

5 1.2 μmol of the title compound was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. A solution containing 0.75% of phenol and 1.75% of sodium chloride (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide
10 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

EXAMPLE 30

A pharmaceutical composition comprising 600 nmol/ml of N^ε⁸²⁹-
15 lithocholoyl human insulin in solution.

1.2 μmol of the title compound was suspended in water (0.8 ml) and dissolved by adjusting the pH value of the solution to 8.5 using 0.2 M sodium hydroxide. To the solution was then added
20 0.8 ml of a stock solution containing 0.75 % cresol and 4% glycerol in water. Finally, the pH value was again adjusted to 8.5 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and
25 transferred aseptically to a cartridge or a vial.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Novo Nordisk A/S
- (B) STREET: Novo Allé
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- (I) TELEX: 37173

(ii) TITLE OF INVENTION: ACYLATED INSULIN

(iii) NUMBER OF SEQUENCES: 49

(iv) CORRESPONDENCE ADDRESS:

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Corporate Patents
- (B) STREET: Novo Alle
- (C) CITY: DK-2880 Bagsvaerd
- (E) COUNTRY: Denmark

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBERS: DK 1044/93 and US 08/190,829
- (B) FILING DATES: 09-SEP-1993 and 02-FEB-1994

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Jørgensen, Dan et al.
- (C) REFERENCE/DOCKET NUMBER: 3985.204-WO,DJ

(ix) TELECOMMUNICATION INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
 1 5 10 15
 Glu Asn Tyr Cys Xaa
 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Val Xaa Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
 1 5 10 15
 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Xaa
 20 25 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 110 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGCTAAGAG ATTCGTTGAC CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60
 TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGCGGGCTG CGTCTAAGCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60
AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCGCCATGG CTAAGAGATT CGTTG 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCTCTAGA GCCTGCGGGC TGCCTCT 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGCTAAGAG ATTCGTTACT CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60
TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

60

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTGCGCATGG CTAAGAGATT CGTTA

25

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 100 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCGGGCTG CGTCTAACCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC

60

AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG

100

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACGTACGTTT TAGAGCCTGC GGGCTGC

27

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTTGGTTG AAGCTTTGTA CTTGGTTTGT GGTGAAAGAG GTTCTTCTA CACTCCAAAG 60
ACTAGAGGTA TCGTTGAA 78

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCTG AAGCTAGATT CGTTAACCAA 60
CAC 63

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 65 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCGA AGCTGAAAGA TTCGTTAACC 60
AACAC 65

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

62

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATATCAA TTTCATACAC	60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	112
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
1 5 10	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
15 20 25	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
30 35 40	
GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC	256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His	
45 50 55	
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC	304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr	
60 65 70 75	
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT	352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr	
80 85 90	
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT AAC TAGACGCAGC	401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	
95 100	
CCGCAGGCTC TAGA	415

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
1 5 10 15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
20 25 30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
35 40 45
Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
50 55 60

63

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80
 Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95
 Tyr Gln Leu Glu Asn Tyr Cys Asn
 100

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TGGTTTTCTT ACTTCGGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAAC TC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAACCTTT TGATGACATT GATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC	60
--	----

64

AATATAAACG ATTA	AAAAGA	ATG	AGA	TTT	CCT	TCA	ATT	TTT	ACT	GCA	GTT	TTA		112		
		Met	Arg	Phe	Pro	Ser	Ile	Phe	Thr	Ala	Val	Leu				
		1					5					10				
TTC	GCA	GCA	TCC	TCC	GCA	TTA	GCT	GCT	CCA	GTC	AAC	ACT	ACA	ACA	GAA	160
Phe	Ala	Ala	Ser	Ser	Ala	Leu	Ala	Ala	Pro	Val	Asn	Thr	Thr	Thr	Glu	
			15					20						25		
GAT	GAA	ACG	GCA	CAA	ATT	CCG	GCT	GAA	GCT	GTC	ATC	GGT	TAC	TCA	GAT	208
Asp	Glu	Thr	Ala	Gln	Ile	Pro	Ala	Glu	Ala	Val	Ile	Gly	Tyr	Ser	Asp	
		30					35					40				
TTA	GAA	GGG	GAT	TTC	GAT	GTT	GCT	GTT	TTG	CCA	TTT	TCC	AAC	AGC	ACA	256
Leu	Glu	Gly	Asp	Phe	Asp	Val	Ala	Val	Leu	Pro	Phe	Ser	Asn	Ser	Thr	
	45					50					55					
AAT	AAC	GGG	TTA	TTG	TTT	ATA	AAT	ACT	ACT	ATT	GCC	AGC	ATT	GCT	GCT	304
Asn	Asn	Gly	Leu	Leu	Phe	Ile	Asn	Thr	Thr	Ile	Ala	Ser	Ile	Ala	Ala	
	60				65					70					75	
AAA	GAA	GAA	GGG	GTA	TCT	TTG	GAT	AAG	AGA	GAA	GTT	AAC	CAA	CAC	TTG	352
Lys	Glu	Glu	Gly	Val	Ser	Leu	Asp	Lys	Arg	Glu	Val	Asn	Gln	His	Leu	
			80						85					90		
TGC	GGT	TCT	CAC	TTG	GTT	GAA	GCT	TTG	TAC	TTG	GTT	TGT	GGT	GAA	AGA	400
Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	
			95					100					105			
GGT	TTC	TTC	TAC	ACT	GAA	AAG	TCT	GAC	GAC	GCT	AAG	GGT	ATC	GTT	GAA	448
Gly	Phe	Phe	Tyr	Thr	Glu	Lys	Ser	Asp	Asp	Ala	Lys	Gly	Ile	Val	Glu	
		110					115					120				
CAA	TGT	TGT	ACT	TCT	ATC	TGT	TCT	TTG	TAC	CAA	TTG	GAA	AAC	TAC	TGT	496
Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	
		125				130					135					
AAC	TAGACGCAGC CCGCAGGCTC TAGA															523
Asn																
140																

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1 5 10 15

65

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
 20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
 35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
 50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65 70 75 80

Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu Cys Gly Ser His Leu
 85 90 95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
 100 105 110

Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
 115 120 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
 130 135 140

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG	120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG	180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG	240
TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG	300
ACGATTTCCTT CTTCCCCATA GAAACCTATT CTCTCTTCAA TTGGTTGTGA ACACGCCAAG	360
AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGACTTTT	420
CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT	480
TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT	523

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATATCAA TTTCATACAC      60
AATATAAAGC ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
          Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
          1             5             10
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
          15             20             25
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
          30             35             40
GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
          45             50             55
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
          60             65             70             75
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
          80             85             90
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
          95             100
CCGCAGGCTC TAGA      415

```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

67

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1           5           10           15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
      20           25           30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
      35           40           45
Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
      50           55           60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
      65           70           75           80
Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
      85           90           95
Tyr Gln Leu Glu Asn Tyr Cys Ala
      100

```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC      120
GACCCGGGTT GGTCA GTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA      180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA      300
GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC      360
AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT      415

```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

68

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATACAA TTTCATACAC      60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                  1             5             10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
              15             20             25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
              30             35             40

GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
              45             50             55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
              60             65             70             75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
              80             85             90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
              95             100

CCGCAGGCTC TAGA      415

```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

69

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1 5 10 15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20 25 30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95

Tyr Gln Leu Glu Asn Tyr Cys Ala
 100

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCA GTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAAA TTTCATACAC	60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	112
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
1 5 10	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
15 20 25	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
30 35 40	
GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC	256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His	
45 50 55	
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC	304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr	
60 65 70 75	
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT	352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr	
80 85 90	
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC	401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly	
95 100	
CCGCAGGCTC TAGA	415

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 104 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
1 5 10 15

71

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20 25 30
 Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45
 Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60
 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80
 Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95
 Tyr Gln Leu Glu Asn Tyr Cys Gly
 100

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTCG TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCACTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCATTCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC      60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                1             5             10
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
                15             20             25
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
                30             35             40
GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
                45             50             55
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
                60             65             70             75
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80             85             90
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
                95             100
CCGCAGGCTC TAGA      415

```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 104 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1             5             10             15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20             25             30

```

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
35 40 45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
50 55 60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65 70 75 80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
85 90 95

Tyr Gln Leu Glu Asn Tyr Cys Gly
100

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCAAGTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS

(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATATCAA TTTCATACAC	60
AATATAAACG ATTAAGAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA	112
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu	
1 5 10	
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA	160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu	
15 20 25	
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT	208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp	
30 35 40	
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA	256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr	
45 50 55	
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT	304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala	
60 65 70 75	
AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA TTC GTT AAC CAA CAC TTG	352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu	
80 85 90	
TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA	400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg	
95 100 105	
GGT TTC TTC TAC ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA	448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu	
110 115 120	
CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT	496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys	
125 130 135	
AAC TAGACGCAGC CCGCAGGCTC TAGA	523
Asn	
140	

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

75

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1 5 10 15
 Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
 20 25 30
 Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
 35 40 45
 Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
 50 55 60
 Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65 70 75 80
 Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
 85 90 95
 Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
 100 105 110
 Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
 115 120 125
 Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
 130 135 140

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG 60
 TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG 120
 TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG 180
 CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCTA AAGCTACAAC GACAAAACGG 240
 TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTGTAACG 300
 ACGATTTCCT CTCCCCATA GAAACCTATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG 360
 AGTGAACCAA CTTGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGAGGTTT 420
 CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT 480

TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT

523

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 409 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..385

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAAA TTTCATACAC	60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	112
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
1 5 10	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
15 20 25	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
30 35 40	
GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC	256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His	
45 50 55	
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC	304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr	
60 65 70 75	
ACT CCT AAG GAA AAG AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC	352
Thr Pro Lys Glu Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile	
80 85 90	
TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC CCGCAGGCTC	405
Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly	
95 100	
TAGA	409

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 amino acids

77

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1           5           10          15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
          20          25          30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
      35          40          45
Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
      50          55          60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Glu Lys
      65          70          75          80
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
          85          90          95
Leu Glu Asn Tyr Cys Gly
          100

```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 409 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC      120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAAC TC TAAGGCCTTC TCAGAGACTA      180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAGAA      300
GATGTGAGGA TTCCTTTTCT CTCCATAGCA ACTTGTTACA ACATGAAGAT AGACAAGAAA      360
CATGGTTAAC CTTTGGATGA CACCAATCTG CGTCGGGCGT CCGAGATCT      409

```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 511 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 77..487

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT	60
ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA	109
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu	
1 5 10	
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA	157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu	
15 20 25	
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT	205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp	
30 35 40	
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA	253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr	
45 50 55	
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT	301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala	
60 65 70 75	
AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG	349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu	
80 85 90	
TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA	397
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg	
95 100 105	
GGT TTC TTC TAC ACT CCA AAG ACT AGA GGT ATC GTT GAA CAA TGT TGT	445
Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys	
110 115 120	
ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGC AAC	487
Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	
125 130 135	
TAGACGCAGC CCGCAGGCTC TAGA	511

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 137 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1             5             10             15
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
      20             25             30
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
      35             40             45
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
      50             55             60
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
      65             70             75             80
Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
      85             90             95
Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
      100            105            110
Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser
      115            120            125
Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
      130            135

```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 511 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```

CTTAAGGTAA GTTCTTATCA AGTTTGTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA      60
TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTA AAAATGA CGTCAAAATA AGCGTCGTAG      120

```

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG 180
 ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA 240
 AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG 300
 ATTTCTTCTT CCCCATAGGT ACCGATTCTC TAAGCAATTG GTTGTGAACA CGCCAAGGGT 360
 GAACCAACTT CGAAACATGA ACCAAACACC ACTTTCTCCA AAGAAGATGT GAGGTTTCTG 420
 ATCTCCATAG CAACTTGTTA CAACATGAAG ATAGACAAGA AACATGGTTA ACCTTTTGAT 480
 GACGTTGATC TGCCTCGGGC GTCCGAGATC T 511

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC 60
 AATATAAACG ATTAAGAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA 112
 Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
 1 5 10
 TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA 160
 Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
 15 20 25
 GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT 208
 Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
 30 35 40
 TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA 256
 Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
 45 50 55
 AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT 304
 Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
 60 65 70 75
 AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG 352
 Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
 80 85 90

TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGC GGT GAA AGA	400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg	
95 100 105	
GGT TTC TTC TAC ACT CCT AAG TCT GAC GAT GCT AAG GGT ATT GTC GAG	448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu	
110 115 120	
CAA TGC TGT ACC TCC ATC TGC TCC TTG TAC CAA TTG GAA AAC TAC TGC	496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys	
125 130 135	
AAC TAGACGCAGC CCGCAGGCTC TAGA	523
Asn	
140	

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser	
1 5 10 15	
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln	
20 25 30	
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe	
35 40 45	
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu	
50 55 60	
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val	
65 70 75 80	
Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu	
85 90 95	
Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr	
100 105 110	
Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser	
115 120 125	
Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	
130 135 140	

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 523 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG      120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG      180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG      240
TAAAGGTTG TCGTGTATTAT TGCCAATAA CAAATATTTA TGATGATAAC GGTGTAACG      300
ACGATTCTT CTCCCCATA GGTACCGATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG      360
GGTGAACCAA CTTCGAAACA TGAACCAAAC GCCACTTTCT CCAAAGAAGA TGTGAGGATT      420
CAGACTGCTA CGATTCCCAT AACAGCTCGT TAGGACATGG AGGTAGACGA GGAACATGGT      480
TAACCTTTTG ATGACGTTGA TCTGCGTCGG GCGTCCGAGA TCT                          523

```

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 535 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 77..511

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```

GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT      60
ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      109
          Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
              1             5             10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
          15             20             25

```

83

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp 30 35 40	205
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr 45 50 55	253
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala 60 65 70 75	301
AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu 80 85 90	349
GCT AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA GCT Ala Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala 95 100 105	397
TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG ACT Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr 110 115 120	445
AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln 125 130 135	493
TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA Leu Glu Asn Tyr Cys Asn 140 145	535

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 145 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser 1 5 10 15	
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln 20 25 30	
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe 35 40 45	
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu 50 55 60	

84

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65 70 75 80
 Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Arg Phe Val Asn
 85 90 95
 Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
 100 105 110
 Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu
 115 120 125
 Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
 130 135 140
 Asn
 145

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 535 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CTTAAGGTAA GTTCTTATCA AGTTTGTCT TCTAATGTT GATAGTTAA GTATGTGTTA	60
TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAATAATGA CGTCAAATA AGCGTCGTAG	120
GAGGCCTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG	180
ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA	240
AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG	300
ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCTA CTTGACTTC GATCTAAGCA	360
ATTGGTTGTG AACACGCCAA GGGTGAACCA ACTTCGAAAC ATGAACCAA CACCACTTTC	420
TCCAAAGAAG ATGTGAGGTT TCTGATCTCC ATAGCAACTT GTTACAACAT GAAGATAGAC	480
AAGAAACATG GTTAACCTTT TGATGACGTT GATCTGCGTC GGGCGTCCGA GATCT	535

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 538 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 77..514

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT	60
ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA	109
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu	
1 5 10	
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA	157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu	
15 20 25	
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT	205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp	
30 35 40	
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA	253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr	
45 50 55	
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT	301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala	
60 65 70 75	
AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA	349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu	
80 85 90	
GCT GAA AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA	397
Ala Glu Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu	
95 100 105	
GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG	445
Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys	
110 115 120	
ACT AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC	493
Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr	
125 130 135	
CAA TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA	538
Gln Leu Glu Asn Tyr Cys Asn	
140 145	

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 146 amino acids

86

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1           5           10           15
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
      20           25           30
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
      35           40           45
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
      50           55           60
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
      65           70           75           80
Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Glu Arg Phe Val
      85           90           95
Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val
      100          105          110
Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val
      115          120          125
Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr
      130          135          140
Cys Asn
145

```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 538 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

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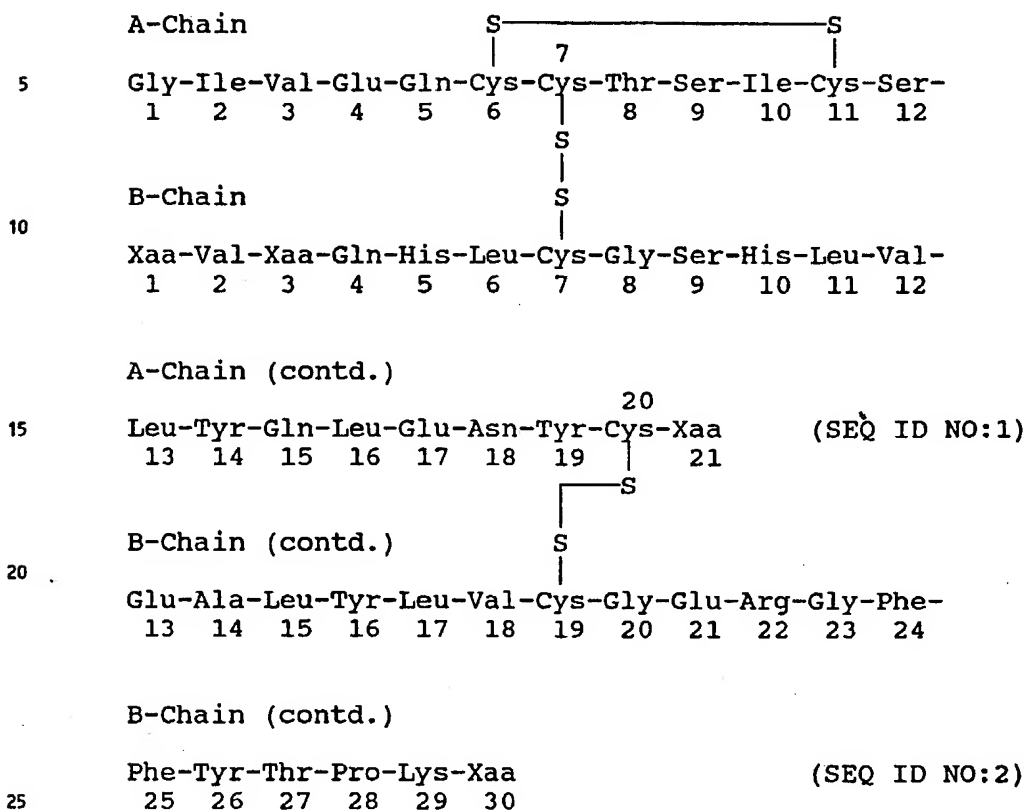
CTTAAGGTAA GTTCTTATCA AGTTTGTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA      60
TATTTGCTAA TTTTCTTACT CTAAGGAAG TAAAAATGA CGTCAAAATA AGCGTCGTAG      120
GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG      180

```

ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA	240
AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG	300
ATTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTGACTTC GACTTTCTAA	360
GCAATTGGTT GTGAACACGC CAAGGGTGAA CCAACTTCGA AACATGAACC AAACACCACT	420
TTCTCCAAAG AAGATGTGAG GTTCTGATC TCCATAGCAA CTTGTTACAA CATGAAGATA	480
GACAAGAAAC ATGGTTAACC TTTTGATGAC GTTGATCTGC GTCGGGCGTC CGAGATCT	538

CLAIMS

1. An insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

30 Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ -amino group of Lys^{B29}, (b) any amino acid residue
 35 which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent ; and
 any Zn²⁺ complexes thereof,

provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn^{2+} complex.

2. The insulin derivative according to claim 1, wherein

5 Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is a non-codable, lipophilic
10 amino acid having from 10 to 24 carbon atoms and an acyl group is bound to the ϵ -amino group of Lys⁸²⁹, wherein the acyl group is an acyl group of a monocarboxylic acid with up to 4 carbon atoms or of a dicarboxylic acid with up to 5 carbon atoms.

3. The insulin derivative according to claim 1, wherein

15 Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is deleted or is any amino acid
20 residue which can be coded for by the genetic code except Lys, Arg and Cys and the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent which comprises at least 6 carbon atoms.

4. The insulin derivative according to claim 2, wherein Xaa at position B30 is selected from the group consisting of α -amino
25 decanoic acid, α -amino dodecanoic acid, α -amino tetradecanoic acid and α -amino hexadecanoic acid.

5. The insulin derivative according to claim 2, wherein the acyl group bound to the ϵ -amino group of Lys⁸²⁹ is selected from the group consisting of formyl, acetyl, propionyl and n-
30 butyryl.

6. The insulin derivative according to claim 2, wherein the acyl group bound to the ϵ -amino group of Lys^{B29} is an acyl group of succinic acid.
7. The insulin derivative according to claim 3, wherein Xaa at position B30 is deleted.
8. The insulin derivative according to claim 3, wherein Xaa at position B30 is Asp, Glu, or Thr.
9. The insulin derivative according to claim 3, wherein the lipophilic substituent bound to the ϵ -amino group of Lys^{B29} is an acyl group derived from a carboxylic acid having at least 6 carbon atoms.
10. The insulin derivative according to claim 9, wherein the acyl group, which may be branched, comprises a main chain of carbon atoms 8 - 24 atoms long.
11. The insulin derivative according to claim 9, wherein the acyl group is an acyl group of a fatty acid having at least 6 carbon atoms.
12. The insulin derivative according to claim 9, wherein the acyl group is an acyl group of a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.
13. The insulin derivative according to claim 9, wherein the acyl group is selected from the group comprising dodecanoic acid, tridecanoic acid and tetradecanoic acid.
14. The insulin derivative according to claim 1, wherein Xaa at position A21 is Ala, Gln, Gly or Ser.
15. The insulin derivative according to claim 1, wherein Xaa at position B3 is Asp, Gln or Thr.

16. The insulin derivative according to claim 1, wherein Xaa at position B1 is deleted.

17. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a
5 therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

18. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a
10 therapeutically effective amount of an insulin derivative according to claim 1, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

19. A method of treating diabetes in a patient in need of such
15 a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

20. A method of treating diabetes in a patient in need of such
20 a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

1/3

NcoI - PCR DNA fragment - XbaI

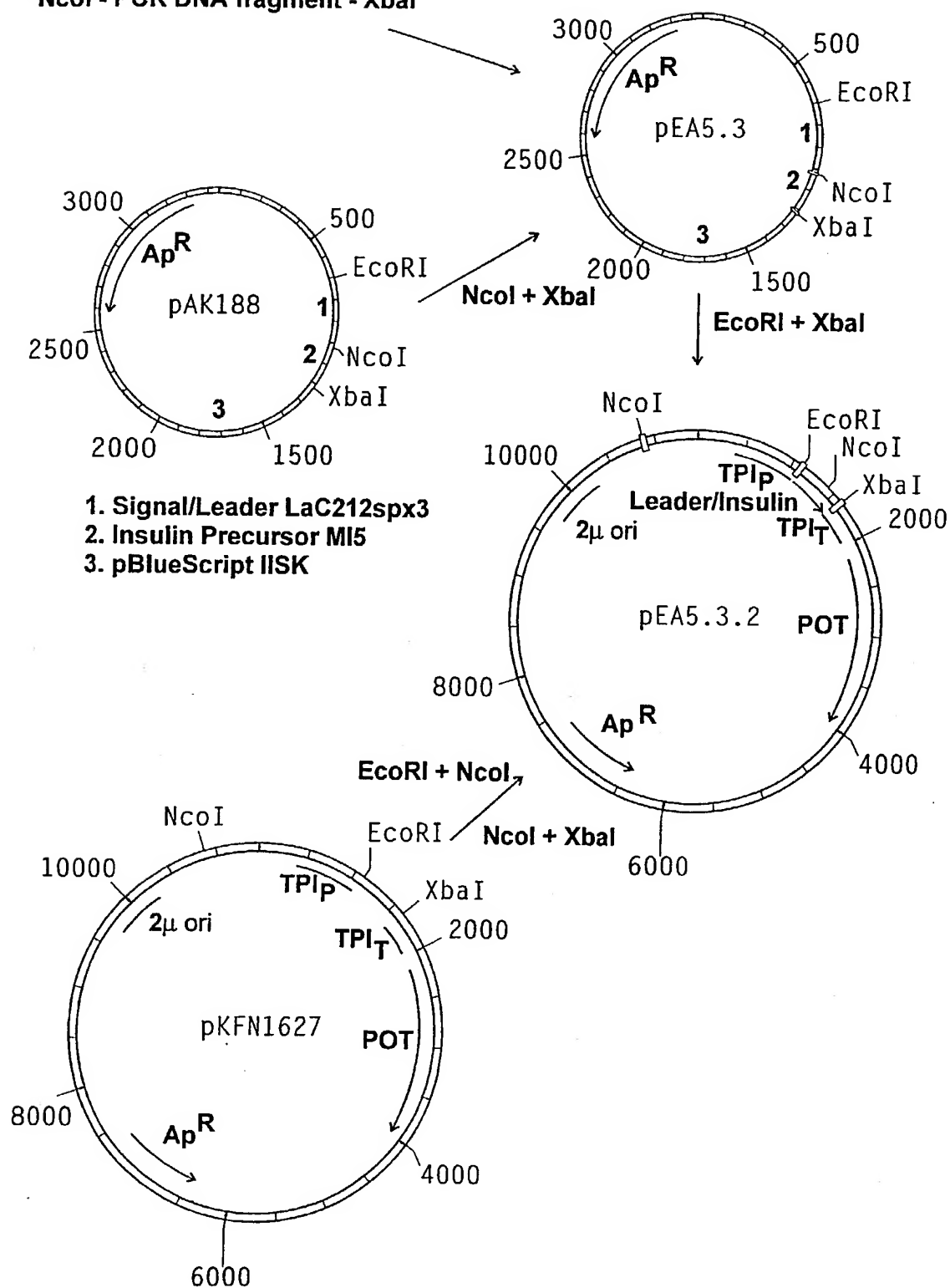


Fig. 1

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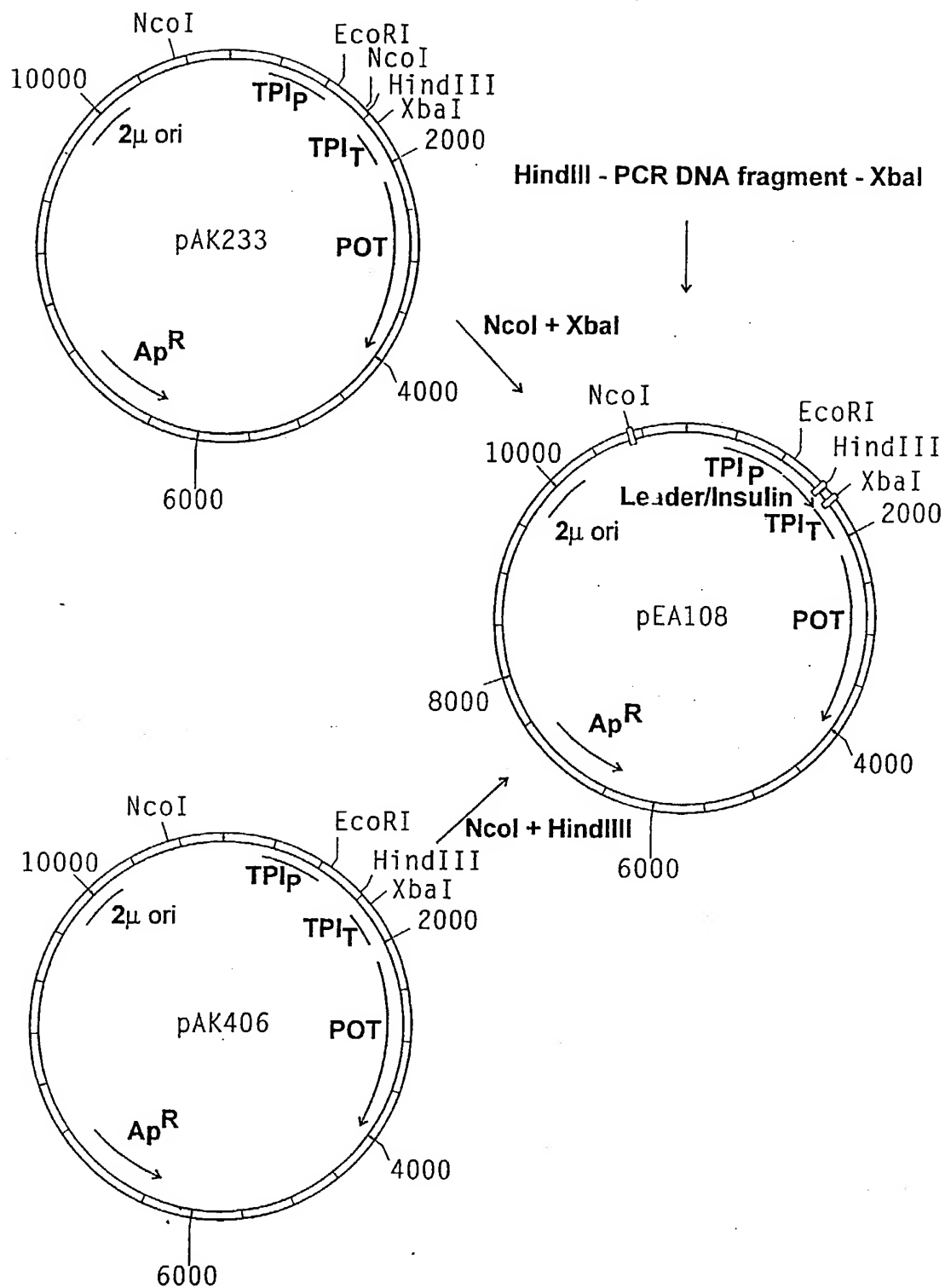


Fig. 2

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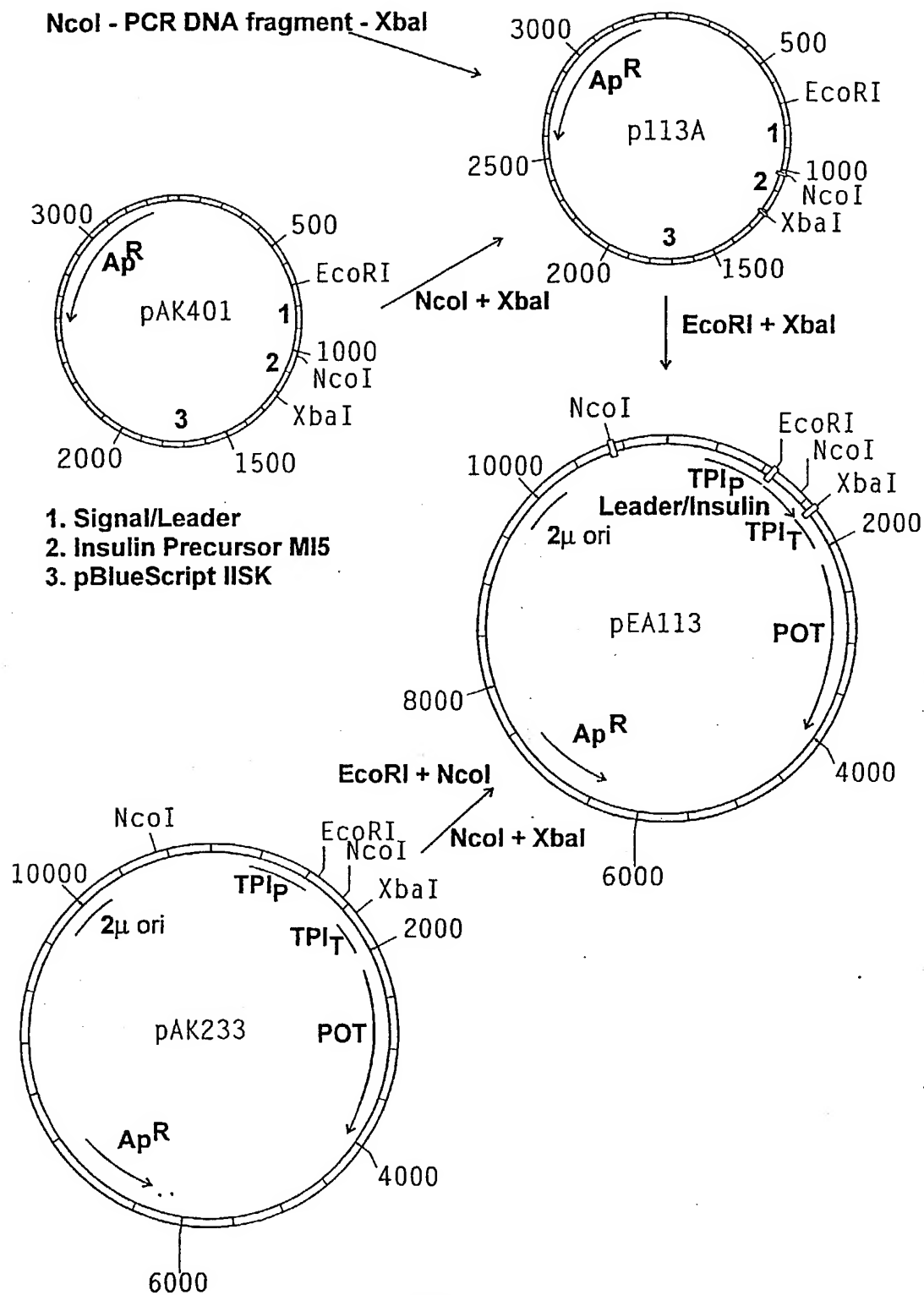


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00347

A. CLASSIFICATION OF SUBJECT MATTER		
IPC6: C07K 14/62, A61K 38/28 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: A61K, C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
MEDLINE, BIOSIS, EMBASE, WPI, CA, CLAIMS, JAPIO		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Patent Abstracts of Japan, Vol 14, No 7, C-673, abstract of JP, A, 1--254699 (KODAMA K.K.), 11 October 1989 (11.10.89) --	1-18
A	US, A, 3823125 (N. H. GRANT ET AL), 9 July 1974 (09.07.74) --	1-18
A	DE, B2, 2209835 (BAYER AG), 29 April 1976 (29.04.76) --	1-18
A	US, A, 3868356 (D. G. SMYTH), 25 February 1975 (25.02.75) --	1-18
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
28 December 1994		05 01-1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Elisabeth Carlborg Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00347

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0127535 (HADASSAH MEDICAL ORGANIZATION), 5 December 1984 (05.12.84) -- -----	1-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00347

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 19, 20
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/11/94

International application No.

PCT/DK 94/00347

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US-A-	3823125	09/07/74	NONE		
DE-B2-	2209835	29/04/76	AT-B-	333987	27/12/76
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US005866538A

United States Patent [19]

Norup et al.

[11] **Patent Number:** 5,866,538[45] **Date of Patent:** Feb. 2, 1999[54] **INSULIN PREPARATIONS CONTAINING
NACL**

5,559,094 9/1996 Brems et al. 514/3

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Langkjær, Klampenborg; Svend
Havelund, Bagsvaerd, all of Denmark**FOREIGN PATENT DOCUMENTS**

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[73] **Assignee:** Novo Nordisk A/S, Bagsvaerd,
Denmark**OTHER PUBLICATIONS**[21] **Appl. No.:** 879,991Brange J. and Langkjoer, L. Insulin Structure and stability.
In: Stability and characterization of protein and peptide
drugs: Case histories, Y.J. Wang, ed., Plenum Press, N.Y., p.
334, Jun. 1993.[22] **Filed:** Jun. 20, 1997Vinita Gupta et al., "Effect of Solvent Additives On the
Thermal Stability of Insulin", Centre for Biotechnology, vol.
70., pp. 209-212.**Related U.S. Application Data**[60] **Provisional application No.** 60/020,927, Jun. 27, 1996.**Foreign Application Priority Data**

Jun. 20, 1996 [DK] Denmark 685/96

[51] **Int. Cl.⁶** A61K 38/28; C07K 14/62[52] **U.S. Cl.** 514/3; 530/389.2; 530/388.24;
530/303; 530/304[58] **Field of Search** 514/3**References Cited****U.S. PATENT DOCUMENTS**

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Primary Examiner—Cecilia J. Tsang*Assistant Examiner*—Michael Borin*Attorney, Agent, or Firm*—Steve T. Zelson, Esq.; Reza
Green, Esq.; Carol E. Rozek, Esq.[57] **ABSTRACT**Insulin preparations of superior chemical stability, compris-
ing human insulin or an analogue or derivative thereof,
glycerol and/or mannitol, and 5 to 100 mM of a halogenide
are disclosed.**17 Claims, No Drawings**

INSULIN PREPARATIONS CONTAINING NACL

INTRODUCTION

This application is a continuation of provisional application Ser. No. 60/020,927, filed Jun. 27, 1996.

The present invention relates to aqueous insulin preparations comprising human insulin or an analogue or derivative thereof, which preparations have superior chemical stability. The invention furthermore relates to parenteral formulations comprising such insulin preparations and to a method for improving the chemical stability of insulin preparations.

BACKGROUND OF THE INVENTION

Diabetes is a general term for disorders in man having excessive urine excretion as in diabetes mellitus and diabetes insipidus. Diabetes mellitus is a metabolic disorder in which the ability to utilize glucose is more or less completely lost. About 2% of all people suffer from diabetes.

Since the introduction of insulin in the 1920's, continuous strides have been made to improve the treatment of diabetes mellitus. To help avoid extreme glycemia levels, diabetic patients often practice multiple injection therapy, whereby insulin is administered with each meal.

In the treatment of diabetes mellitus, many varieties of insulin preparations have been suggested and used, such as regular insulin, Semilente® insulin, isophane insulin, insulin zinc suspensions, protamine zinc insulin, and Ultralente® insulin. As diabetic patients are treated with insulin for several decades, there is a major need for safe and life quality improving insulin preparations. Some of the commercial available insulin preparations are characterized by a fast onset of action and other preparations have a relatively slow onset but show a more or less prolonged action. Fast acting insulin preparations are usually solutions of insulin, while retarded acting insulin preparations can be suspensions containing insulin in crystalline and/or amorphous form precipitated by addition of zinc salts alone or by addition of protamine or by a combination of both. In addition, some patients are using preparations having both a fast onset of action and a more prolonged action. Such a preparation may be an insulin solution wherein protamine insulin crystals are suspended. Some patients do themselves prepare the final preparation by mixing an insulin solution with a suspension preparation in the ratio desired by the patient in question.

Human insulin consists of two polypeptide chains, the so-called A and B chains which contain 21 and 30 amino acids, respectively. The A and B chains are interconnected by two cystine disulphide bridges. Insulin from most other species has a similar construction, but may not contain the same amino acids at the positions corresponding in the chains as in human insulin.

The development of the process known as genetic engineering has made it possible easily to prepare a great variety of insulin compounds being analogous to human insulin. In these insulin analogues, one or more of the amino acids have been substituted with other amino acids which can be coded for by the nucleotide sequences. As human insulin, as explained above, contains 51 amino acid residues, it is obvious that a large number of insulin analogues is possible and, in fact, a great variety of analogues with interesting properties have been prepared. In human insulin solutions with a concentration of interest for injection preparations, the insulin molecule is present in associated form as a

hexamer (Brange et al. *Diabetes Care* 13, (1990), 923-954). After subcutaneous injection, it is believed that the rate of absorption by the blood stream is dependent of the size of the molecule, and it has been found that insulin analogues with amino acid substitutions which counteract or inhibit this hexamer formation have an unusual fast onset of action (Brange et al.: *Ibid*). This is of great therapeutic value for the diabetic patient.

Pharmaceutical preparations which are based on analogues of human insulin have e.g. been presented by Heinemann et al., Lutterman et al. and Wiefels et al. at the "Frontiers in Insulin Pharmacology" International Symposium in Hamburg, 1992.

Furthermore, U.S. Pat. No. 5,474,978 discloses a rapid acting parenteral formulation comprising a human insulin analogue hexamer complex consisting of six monomeric insulin analogues, zinc ions and at least three molecules of a phenolic derivative.

Normally, insulin preparations are administered by subcutaneous injection. What is important for the patient, is the action profile of the insulin preparation which is the action of insulin on the glucose metabolism as a function of the time from the injection. In this profile, inter alia, the time for the onset, the maximum value and the total duration of action are important. A variety of insulin preparations with different action profiles are desired and requested by the patients. One patient may, on the same day, use insulin preparations with very different action profiles. The action profile requested is, for example, depending on the time of the day and the amount and composition of any meal eaten by the patient.

Equally important for the patient is the chemical stability of the insulin preparations, especially due to the abundant use of pen-like injection devices such as devices which contain Penfill® cartridges, in which an insulin preparation is stored until the entire cartridge is empty. This may last for at least 1 to 2 weeks for devices containing 1.5-3.0 ml cartridges. During storage, covalent chemical changes in the insulin structure occur. This may lead to formation of molecules which are less active and potentially immunogenic such as deamidation products and higher molecular weight transformation products (dimers, polymers, etc.). A comprehensive study on the chemical stability of insulin is given in by Jens Brange in "Stability of Insulin", Kluwer Academic Publishers, 1994.

Acta Pharmaceutica Nordica 4(4), 1992, pp. 149-158 discloses insulin preparations in which the sodium chloride concentration has been varied in the range of 0 to 250 mM. However, the major part of the preparations, including all preparations which additionally comprises glycerol, contains a rather high amount of sodium chloride, i.e. 0.7% corresponding approximately to a concentration of 120 mM. It is stated in this document that whereas sodium chloride generally has a stabilizing effect on insulin preparations, glycerol and glucose lead to increased chemical deterioration.

Surprisingly, however, it has now been shown that insulin preparations of superior chemical stability can be obtained in the presence of glycerol and/or mannitol and rather low halogenide concentrations.

DESCRIPTION OF THE INVENTION

By "analogue of human insulin" as used herein is meant human insulin in which one or more amino acids have been deleted and/or replaced by other amino acids, including non-codeable amino acids, or human insulin comprising additional amino acids, i.e. more than 51 amino acids.

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By "derivative of human insulin" as used herein is meant human insulin or an analogue thereof in which at least one organic substituent is bound to one or more of the amino acids.

In the present context the unit "U" corresponds to 6 nmol. The present invention relates to an aqueous insulin preparation comprising:

human insulin, an analogue thereof and/or a derivative thereof,

glycerol and/or mannitol, and

5 to 100 mM of a halogenide.

The above insulin preparation has a high chemical stability which e.g. is reflected in a reduction in the formation of dimers and polymers and desamido insulins after storage. Furthermore, the physical stability is not deteriorated by the presence of the rather low amount of halogenide, and the insulin does not precipitate by long-term storage of the insulin preparations.

The halogenide is preferably an alkali or alkaline earth halogenide, more preferably a chloride such as sodium chloride.

Glycerol and/or mannitol is preferably present in an amount corresponding to a concentration of 100 to 250 mM, more preferably 140 to 250 mM, even more preferably 160 to 200 mM.

The present invention is particularly advantageous in connection with preparations comprising analogues and/or derivatives of human insulin. Thus, the insulin preparation according to the invention preferably comprises one or more fast-acting analogues of human insulin, in particular analogues wherein position B28 is Asp, Lys, Leu, Val or Ala and position B29 is Lys or Pro; or des(B28-B30), des(B27) or des(B30) human insulin. The insulin analogue is preferably selected from analogues of human insulin wherein position B28 is Asp or Lys, and position B29 is Lys or Pro. The most preferred analogues are Asp^{B28} human insulin or Lys^{B28}Pro^{B29} human insulin.

In this embodiment, the insulin preparation preferably comprises 5 to 60 mM, more preferably 5 to 40 mM, of a halogenide.

In another embodiment the insulin preparation according to the invention comprises an insulin derivative having a protracted profile of action such as insulins having one or more lipophilic substituents. The preferred lipophilic insulins are acylated insulins, including those described in WO 95/07931 (Novo Nordisk A/S), e.g. human insulin derivatives wherein the ϵ -amino group of Lys^{B29} contains an acyl substituent which comprises at least 6 carbon atoms.

The preferred insulins derivatives are the following:

B29-N^ε-myristoyl-des(B30) human insulin, B29-N^ε-palmitoyl-des(B30) human insulin, B29-N^ε-myristoyl human insulin, B29-N^ε-palmitoyl human insulin, B28-N^ε-myristoyl Lys^{B28}Pro^{B29} human insulin, B28-N^ε-palmitoyl Lys^{B28}Pro^{B29} human insulin, B30-N^ε-myristoyl-Thr^{B29}Lys^{B30} human insulin, B30-N^ε-palmitoyl-Thr^{B29}Lys^{B30} human insulin, B29-N^ε-(N-palmitoyl-γ-glutamyl)-des(B30) human insulin, B29-N^ε-(N-lithocholyl-γ-glutamyl)-des(B30) human insulin and B29-N^ε-(ω-carboxyheptadecanoyl)-des(B30) human insulin, B29-N^ε-(ω-carboxyheptadecanoyl) human insulin; the most preferred being B29-N^ε-myristoyl-des(B30) human insulin.

In this embodiment, the insulin preparation preferably comprises 10 to 100 mM, more preferably 10 to 70 mM, of a halogenide.

In a particular embodiment, the insulin preparation of the invention comprises an insulin analogue as well as an insulin derivative.

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In a preferred embodiment of the invention the insulin preparation comprises:

60 to 3000 nmol/ml, preferably 240 to 1200 nmol/ml, of human insulin or insulin analogue or derivative,

10 to 40 μg Zn/100 U insulin, preferably 10 to 26 μg Zn/100 U insulin, and

0 to 5 mg/ml, preferably 0 to 4 mg/ml, of a phenolic compound.

As a phenolic compound, 0.5 to 4.0 mg/ml, preferably 0.6 to 4.0 mg/ml, of m-cresol or 0.5 to 4.0 mg/ml, preferably 1.4 to 4.0 mg/ml, of phenol, or a mixture thereof, is advantageously employed.

The insulin preparation of the present invention may furthermore contain other ingredients common to insulin preparations, for example zinc complexing agents such as citrate, and phosphate buffers.

The present invention furthermore relates to a parenteral pharmaceutical formulation comprising an insulin preparation of the invention.

Moreover, the present invention is concerned with a method for improving the chemical stability of an insulin preparation comprising human insulin or an analogue or a derivative thereof, which method comprises adding glycerol and/or mannitol and 5 to 100 mM of a halogenide to said preparation.

The invention is further illustrated by the following examples which, however, are not to be construed as limiting.

EXAMPLE I

Solutions containing 100 U/ml Asp^{B28} human insulin, 2.6 mg/ml phenol, 16 mg/ml glycerol and varying amounts of Zn and sodium chloride were prepared. The pH was varied in the range of 7.2 to 7.5. Stability data after 4 weeks at 37° C. are presented in the following Table 1.

TABLE 1

μg Zn/100 U insulin	NaCl (mM)	pH	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
13.1	0	7.2	3.44	1.35
		7.5	3.57	1.36
	5	7.2	3.48	1.53
		7.5	3.31	1.49
16.3	20	7.2	2.54	1.72
		7.5	2.47	1.26
	0	7.2	3.35	1.44
		7.4	3.41	1.46
19.6	5	7.2	1.74	0.95
		7.5	2.58	1.38
	20	7.2	1.91	1.05
		7.5	2.00	1.31
22.8	0	7.2	3.07	1.57
		7.5	2.85	1.80
	5	7.2	2.71	1.36
		7.5	2.24	1.46
55	20	7.2	1.56	1.15
		7.5	1.68	1.13
	0	7.2	2.71	2.52
		7.5	2.34	1.45
60	5	7.2	2.18	1.95
		7.5	1.90	1.19
	20	7.2	1.51	1.05
		7.5	1.46	1.09

EXAMPLE II

Insulin preparations containing dissolved Asp^{B28} human insulin with varying concentrations of sodium chloride was prepared in the following way:

370.4 mg AspB28 human insulin was dissolved in water by adding 1.6 ml 0.2N HCl and 49 μ l zinc chloride solution (40 mg Zn/ml). 40 g of a solution containing 40 mg/ml glycerol, 3.75 mg/g phenol and 4.30 mg/g m-cresol was added to the insulin solution while mixing. 20 g of a solution containing a) 12.0 mg/g disodium phosphate dihydrate+5 μ l/g 2N sodium hydroxide, b) 12.0 mg/g disodium phosphate dihydrate+5 μ l/g 2N sodium hydroxide+5 mg/g sodium chloride or c) 12.0 mg/g disodium phosphate dihydrate+5 μ l/g 2N sodium hydroxide+10 mg/g sodium chloride was added while mixing. pH was adjusted to pH 7.40 \pm 0.05 and water added up to 100 ml. The Asp^{B28} Human insulin preparations were introduced into Penfill® cartridges and subjected to stability tests at 25° C. and 37° C. The stability data obtained at the two different temperatures and at a phosphate concentration of 13.5 mM, 19.6 μ g Zn/100 U insulin and pH=7.4 are summarized in Table 2.

TABLE 2

NaCl added (mM)	Total conc. of Cl ⁻ (mM)	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
Data after 8 weeks at 37° C.			
0	4.4	7.0	1.86
17	20.8	4.2	1.29
34	37.8	3.5	1.07
Data after 8 months at 25° C.			
0	4.4	6.4	1.0
17	20.8	4.1	0.8
34	37.8	3.7	0.8

EXAMPLE III

Insulin preparations containing dissolved Asp^{B28} human insulin with varying concentrations of sodium chloride was prepared in the following way:

369.4 mg Asp^{B28} human insulin was dissolved in water by adding 1.6 ml 0.2N HCl and 49 μ l zinc chloride solution (40 mg Zn/ml). 40 g of a solution containing 40 mg/g glycerol, 3.75 mg/g phenol and 4.30 mg/g m-cresol was added to the solution while mixing. 10 g of a solution containing 24.0 mg/g disodium phosphate dihydrate and 11 μ l/g 2N sodium hydroxide was added while mixing. Finally varying amounts (0 g to 4.38 g) of a solution containing 40 mg/g sodium chloride were added while mixing up to a sodium chloride concentration mentioned in Table 4. pH was adjusted to 7.40 \pm 0.05 and water added up to 100 ml. The Asp^{B28} Human insulin preparations were introduced into Penfill® cartridges and subjected to stability tests at 25° C. and 37° C. The stability data obtained at the two different temperatures and at a phosphate concentration of 13.5 mM are summarized in Table 3.

TABLE 3

NaCl added (mM)	Total conc. of Cl ⁻ (mM)	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
Stability data after 6 weeks at 37° C.			
5	8.5	4.1	0.99
12.5	16.3	3.6	0.92
20	23.8	3.0	0.87
25	28.8	3.0	0.82
30	33.8	2.8	0.80

TABLE 3-continued

NaCl added (mM)	Total conc. of Cl ⁻ (mM)	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
Stability data after 12 weeks at 25° C.			
0	3.8	2.7	0.36
5	8.5	2.3	0.32
12.5	16.3	1.8	0.39
20	23.8	1.7	0.39
25	28.8	1.8	0.38
30	33.8	1.7	0.38

EXAMPLE IV

Insulin preparations containing dissolved Asp^{B28} human insulin with varying concentrations of phosphate and sodium chloride was prepared in the following way:

375.7 mg Asp^{B28} human insulin was dissolved in water by adding 1.6 ml 0.2N HCl and 49 μ l zinc chloride solution (40 mg Zn/ml). 20 g of a solution containing 80 mg/g glycerol, 7.50 mg/g phenol and 8.60 mg/g m-cresol was added to the solution while mixing. Varying amounts (3.71 g to 6.71 g) of a solution containing 24.0 mg/g disodium phosphate dihydrate and 11 μ l/g 2N sodium hydroxide was added while mixing, finally varying amounts (0 g to 3.65 g) of a solution containing 40 mg/g sodium chloride were added while mixing so as to obtain a sodium chloride concentration mentioned in table 6. pH was adjusted to pH 7.40 \pm 0.05 and water added up to 100 ml. The Asp^{B28} Human insulin preparations were introduced into Penfill® cartridges and subjected to stability tests at 25° C. and 37° C. The stability data at the two different temperatures and three different phosphate concentrations and at 19.6 μ g Zn/100 U insulin and pH=7.4 are summarized in Tables 4, 5 and 6.

TABLE 4

NaCl added (mM)	Total conc. of Cl ⁻ (mM)	Phosphate conc. (mM)	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
Data after 6 weeks at 37° C.				
0	3.8	5	4.7	1.4
5	8.8	5	3.7	1.3
10	13.8	5	3.4	1.2
15	18.8	5	3.1	1.1
20	23.8	5	2.7	1.1
25	28.8	5	3.0	0.9
Data after 12 weeks at 25° C.				
0	3.8	5	2.2	0.5
5	8.8	5	1.7	0.4
10	13.8	5	1.5	0.4
15	18.8	5	1.4	0.4
20	23.8	5	1.3	0.4
25	28.8	5	1.3	0.4

TABLE 5

NaCl added (mM)	Total conc. of Cl ⁻ (mM)	Phosphate conc. (mM)	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
Data after 6 weeks at 37° C.				
0	3.8	7	4.3	1.2
5	8.8	7	3.6	1.2

TABLE 5-continued

NaCl added (mM)	Total conc. of Cl ⁻ (mM)	Phosphate conc. (mM)	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
10	13.8	7	3.1	1.1
15	18.8	7	3.1	1.0
20	23.8	7	2.9	1.0
25	28.8	7	2.8	1.1
Data after 12 weeks at 25° C.				
0	3.8	7	2.0	0.5
5	8.8	7	1.7	0.4
10	13.8	7	1.4	0.4
15	18.8	7	1.5	0.4
20	23.8	7	1.4	0.4
25	28.8	7	1.3	0.4

TABLE 6

NaCl added (mM)	Total conc. of Cl ⁻ (mM)	Phosphate conc. (mM)	Asp ^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
Data after 6 weeks at 37° C.				
0	3.8	9	4.9	1.2
5	8.8	9	4.0	1.1
10	13.8	9	3.7	1.0
15	18.8	9	3.5	1.0
20	23.8	9	3.5	1.0
25	28.8	9	3.1	0.9
Data after 12 weeks at 25° C.				
0	3.8	9	n.d.	0.4
5	8.8	9	1.8	0.4
10	13.8	9	1.5	0.4
15	18.8	9	1.5	0.4
20	23.8	9	1.6	0.4
25	28.8	9	1.4	0.4

EXAMPLE V

Solutions containing 0.6 mM B29-N^ε-myristoyl-des(B30) human insulin, 1.5 or 4.0 mg/ml phenol, 5 mM sodium phosphate, 13.1 μg/ml Zn, and varying amounts of sodium chloride and mannitol were prepared. pH was adjusted to 7.4. Stability data (formation of dimers and polymers) after storage at 25° C. for 13 weeks or 37° C. for 8 weeks are presented in the following table 7.

TABLE 7

NaCl (mM)	Mannitol (mg/ml)	Phenol 1.5 mg/ml	Phenol 4.0 mg/ml
Di- & polymers (%) formed after 8 weeks at 37° C.			
20	31	0.77	0.77
50	22	0.71	0.71
75	13	0.65	0.70
100	5	0.66	0.68
Di- & polymers (%) formed after 13 weeks at 25° C.			
20	31	0.40	0.42
50	22	0.35	0.37
75	13	0.34	0.39
100	5	0.31	0.37

EXAMPLE VI

Solutions containing 0.6 mM B29-N^ε-myristoyl des(B30) human insulin, 1.5 mg/ml phenol and 1.72 mg/ml m-cresol,

16 mg/ml glycerol or 36 mg/ml mannitol, 13.1 μg/ml Zn, 7 mM sodium phosphate and varying amounts of sodium chloride were prepared. pH was adjusted to 7.5. Stability data (formation of dimers and polymers) after storage at 25° C. for 13 weeks or 37° C. for 8 weeks are presented in the following table 8.

TABLE 8

NaCl (mM)	Glycerol 16 mg/ml	Mannitol 36 mg/ml
Di- & polymers (%) formed after 8 weeks at 37° C.		
5	2.55	2.28
10	2.25	1.90
15	1.82	1.61
30	1.83	n.d.
40	1.78	1.56
50	1.68	n.d.
Di- & polymers (%) formed after 13 weeks at 25° C.		
5	1.08	1.05
10	0.98	0.84
20	0.80	0.71
30	0.80	n.d.
40	0.79	0.70
50	0.72	n.d.

We claim:

1. A pharmaceutical formulation comprising:

a polypeptide selected from the group consisting of human insulin, an analogue thereof, a derivative thereof, and combinations of any of the foregoing; glycerol, mannitol, or glycerol and mannitol; and 5 to 100 mM of a halogenide.

2. A pharmaceutical formulation according to claim 1, wherein the halogenide is an alkali or alkaline earth halogenide.

3. A pharmaceutical formulation according to claim 1, wherein said glycerol or mannitol is present at a concentration of 100 to 250 mM.

4. A pharmaceutical formulation according to claim 1, wherein said polypeptide is an analogue of human insulin selected from the group consisting of: (i) an analogue wherein position B28 is Asp, Lys, Leu, Val or Ala and position B29 is Lys or Pro; and (ii) des(B28-B30), des(B27) or des(B30) human insulin.

5. A pharmaceutical formulation according to claim 4, wherein said polypeptide is an analogue of human insulin wherein position B28 is Asp or Lys, and position B29 is Lys or Pro.

6. A pharmaceutical formulation according to claim 4, wherein said polypeptide is des(B30) human insulin.

7. A pharmaceutical formulation according to claim 1, wherein said halogenide is present at a concentration of 5 to 60 mM.

8. A pharmaceutical formulation according to claim 1, wherein said polypeptide is a derivative of human insulin having one or more lipophilic substituents.

9. A pharmaceutical formulation according to claim 8, wherein the insulin derivative is selected from the group consisting of B29-N^ε-myristoyl-des(B30) human insulin, B29-N^ε-palmitoyl-des(B30) human insulin, B29-N^ε-myristoyl human insulin, B29-N^ε-palmitoyl human insulin, B28-N^ε-myristoyl Lys^{B28}Pro^{B29} human insulin, B28-N^ε-palmitoyl-Lys^{B28}Pro^{B29} human insulin, B30-N^ε-myristoyl-Thr^{B29}Lys^{B30} human insulin, B30-N^ε-palmitoyl-Thr^{B29}Lys^{B30} human insulin, B29-N^ε-(N-palmitoyl-γ-glutamyl)-des(B30) human insulin, B29-N^ε-(N-lithocholyl-

γ -glutamyl)-des(B30) human insulin, B29-N^ε-(ω -carboxyheptadecanoyl)-des(B30) human insulin and B29-N^ε-(ω -carboxyheptadecanoyl) human insulin.

10. A pharmaceutical formulation according to claim 9, wherein the insulin derivative is B29-N^ε-myristoyl-des (B30) human insulin.

11. A pharmaceutical formulation according to claim 8, wherein said halogenide is present at a concentration of 10 to 100 mM.

12. A pharmaceutical formulation according to claim 1, comprising an insulin analogue as well as an insulin derivative.

13. A pharmaceutical formulation according to claim 1, wherein said polypeptide is present at a concentration of 60 to 3000 nmol/ml.

14. A pharmaceutical formulation according to claim 1, further comprising:

10 to 40 ug Zn/100 U insulin.

15. A pharmaceutical formulation according to claim 1, further comprising:

0 to 5 mg/ml of a phenolic compound.

16. A pharmaceutical formulation according to claim 15, comprising:

0.5 to 4.0 mg/ml of m-cresol or 0.5 to 4.0 mg/ml of phenol, or a mixture thereof.

17. A pharmaceutical formulation according to claim 1, wherein the halogenide is sodium chloride.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,866,538

DATED : February 2, 1999

INVENTOR(S) : Norup et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 1, line 22: delete "continuos" and insert --continuous--

Signed and Scaled this
Fifteenth Day of June, 1999

Attest:



Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks



US006011007A

United States Patent [19]
Havelund et al.

[11] **Patent Number:** **6,011,007**
 [45] **Date of Patent:** ***Jan. 4, 2000**

[54] **ACYLATED INSULIN**

[75] **Inventors:** Svend Havelund, Bagsvaerd; John Halstrom, Hundested; Ib Jonassen, Valby; Asger Sloth Andersen, Frederiksberg; Jan Markussen, Herlev, all of Denmark

[73] **Assignee:** Novo Nordisk A/S, Bagsvaerd, Denmark

[*] **Notice:** This patent is subject to a terminal disclaimer.

[21] **Appl. No.:** 08/975,365

[22] **Filed:** Nov. 20, 1997

Related U.S. Application Data

[62] Continuation-in-part of application No. 08/400,256, Mar. 8, 1995, Pat. No. 5,750,497, which is a continuation-in-part of application No. 08/190,829, filed as application No. PCT/DK94/00347, Sep. 16, 1994, abandoned.

[30] Foreign Application Priority Data

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[51] **Int. Cl.⁷** C07K 14/62; A61K 38/28

[52] **U.S. Cl.** 514/3; 514/866; 530/303; 530/304

[58] **Field of Search** 530/303.4; 514/24, 514/12, 866

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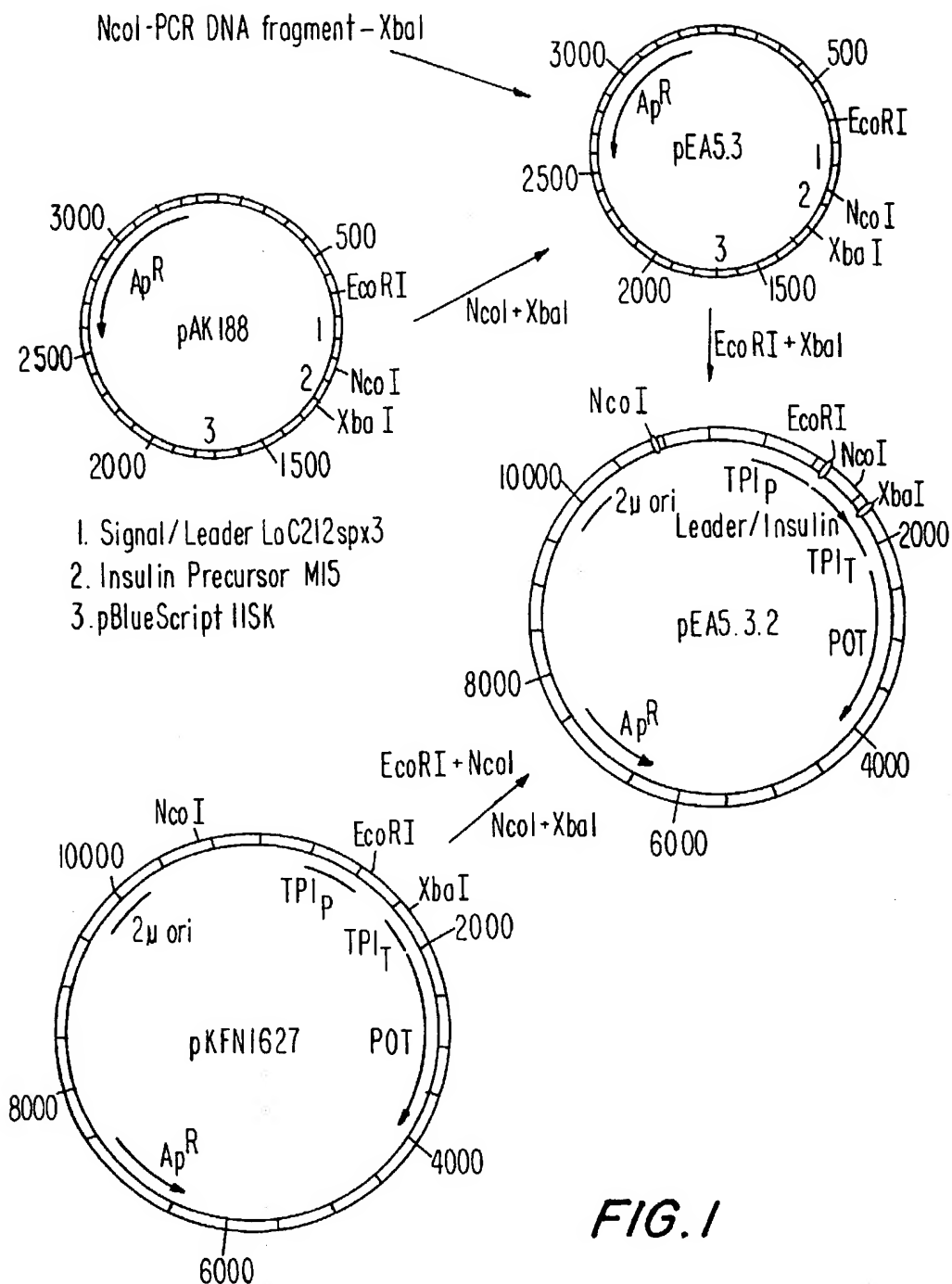
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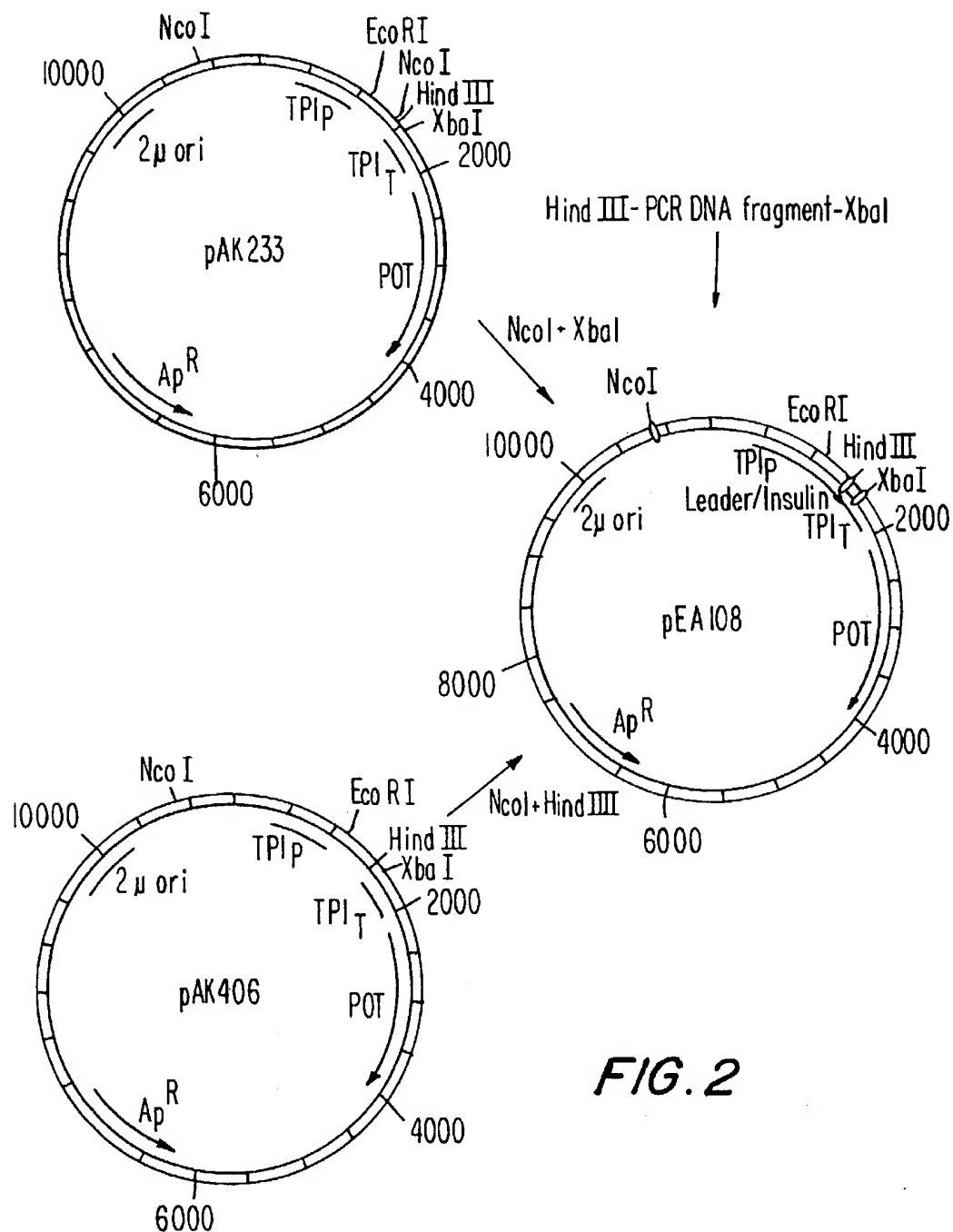
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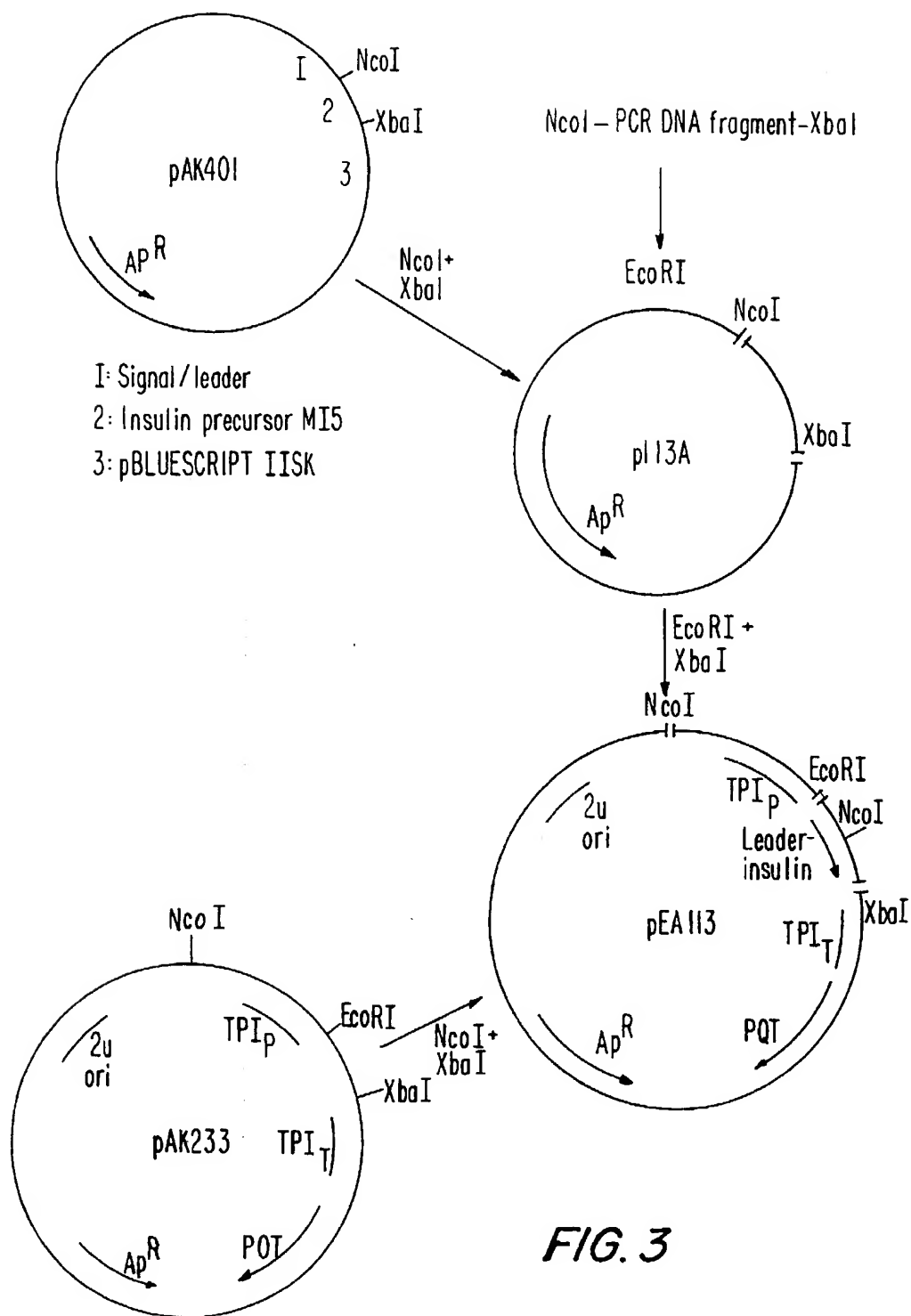
[57] ABSTRACT

The present invention relates to protracted human insulin derivatives in which the A21 and the B3 amino acid residues are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the B30 amino acid residue is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ε-amino group of Lys^{B29}; or (b) the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in any of which cases the ε-amino group of Lys^{B29} has a lipophilic substituent; and any Zn²⁺ complexes thereof with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is present, then the insulin derivative is always present as a Zn²⁺ complex.

115 Claims, 3 Drawing Sheets



**FIG. 2**

**FIG. 3**

ACYLATED INSULIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 08/400,256 filed Mar. 8, 1995 now U.S. Pat. No. 5,750,997, which is a continuation-in-part of Ser. No. 08/190,829 filed Feb. 2, 1994, now abandoned, and Ser. No. PCT/DK94/00347 filed Sep. 16, 1994, now abandoned, the contents of which are fully incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the requirement related to meals.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be immunogenic in man and that their use for medical purposes may lead to formation of antibodies (Samuel et al., Studies on the immunogenicity of protamines in humans and experimental animals by means of a micro-complement fixation test, Clin. Exp. Immunol. 33, pp. 252-260 (1978)).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz et al., Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologica 25, pp. 322-324 (1983)). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback with these solutions is that the particle size distribution of the precipitate formed in the tissue on injection, and thus the timing of the medication, depends on the blood flow at the injection site and other parameters in a somewhat unpredictable manner. A further drawback is that the solid particles of the insulin may act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction of these complexes is only intermediate and the bioavailability is reduced.

Human insulin has three primary amino groups: the N-terminal group of the A-chain and of the B-chain and the ϵ -amino group of Lys^{B29}. Several insulin derivatives which are substituted in one or more of these groups are known in the prior art. Thus, U.S. Pat. No. 3,528,960 (Eli Lilly) relates to N-carboxyaroyl insulins in which one, two or three primary amino groups of the insulin molecule has a carboxyaroyl group. No specifically N^{B29}-substituted insulins are disclosed.

According to GB Patent No. 1,492,997 (Nat. Res. Dev. Corp.), it has been found that insulin with a carbamyl substitution at N^{B29} has an improved profile of hypoglycaemic effect.

JP laid-open patent application No. 1-254699 (Kodama Co., Ltd.) discloses insulin wherein a fatty acid is bound to the amino group of Phe^{B1} or to the ϵ -amino group of Lys^{B29} or to both of these. The stated purpose of the derivatisation is to obtain a pharmacologically acceptable, stable insulin preparation.

Insulins, which in the B30 position have an amino acid having at least five carbon atoms which cannot necessarily be coded for by a triplet of nucleotides, are described in JP laid-open patent application No. 57-067548 (Shionogi). The insulin analogues are claimed to be useful in the treatment of diabetes mellitus, particularly in patients who are insulin resistant due to generation of bovine or swine insulin antibodies.

By "insulin derivative" as used herein is meant a compound having a molecular structure similar to that of human insulin including the disulfide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulfide bridge between Cys^{A6} and Cys^{A11}, and which have insulin activity.

However, there still is a need for protracted injectable insulin compositions which are solutions and contain insulins which stay in solution after injection and possess minimal inflammatory and immunogenic properties.

One object of the present invention is to provide human insulin derivatives, with a protracted profile of action, which are soluble at physiological pH values.

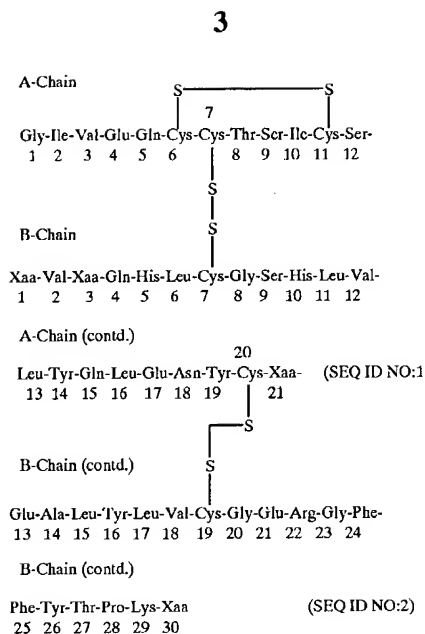
Another object of the present invention is to provide a pharmaceutical composition comprising the human insulin derivatives according to the invention.

It is a further object of the invention to provide a method of making the human insulin derivatives of the invention.

SUMMARY OF THE INVENTION

Surprisingly, it has turned out that certain human insulin derivatives, wherein the ϵ -amino group of Lys^{B29} has a lipophilic substituent, have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to an insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ -amino group of Lys^{B29}, (b) any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent; and any Zn²⁺ complexes thereof, provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn²⁺ complex.

In one preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn²⁺ ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is not deleted, then 2-4 Zn²⁺ ions are bound to each hexamer of the insulin derivative.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys, with the proviso that if the B30 amino acid residue is Ala or Thr, then at least one of the residues A21 and B3 is different from Asn; Phe^{B1} may be deleted; and the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms.

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In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn²⁺ ions are bound to each insulin hexamer.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Glu.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic amino acid having at least 10 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic α -amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a straight chain, saturated, aliphatic α -amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is D- or L-N^ε-dodecanoyllysine.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino decanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino undecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino dodecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino tridecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino tetradecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino pentadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino hexadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is an α -amino acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ala.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gly.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ser.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a carboxylic acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group, branched or unbranched, which corresponds to a carboxylic acid having a chain of carbon atoms 8 to 24 atoms long.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a fatty acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 8 to 12 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 10 to 16 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an oligo oxyethylene group comprising up to 10, preferably up to 5, oxyethylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has a lipophilic substituent which is an oligo oxypropylene group comprising up to 10, preferably up to 5, oxypropylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 2 Zn²⁺ ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 3 Zn²⁺ ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 4 Zn²⁺ ions.

In another preferred embodiment, the invention relates to the use of a human insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a

therapeutically effective amount of a human insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at physiological pH values.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at pH values in the interval from about 6.5 to about 8.5.

In another preferred embodiment, the invention relates to a protracted pharmaceutical composition comprising a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a pharmaceutical composition which is a solution containing from about 120 nmol/ml to about 1200 nmol/ml, preferably about 600 nmol/ml of a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

Examples of preferred human insulin derivatives according to the present invention in which no Zn²⁺ ions are bound are the following:

N^{εB29}-tridecanoyl des(B30) human insulin,
 N^{εB29}-tetradecanoyl des(B30) human insulin,
 N^{εB29}-decanoyl des(B30) human insulin,
 N^{εB29}-dodecanoyl des(B30) human insulin,
 N^{εB29}-tridecanoyl Gly^{A21} des(B30) human insulin,
 N^{εB29}-tetradecanoyl Gly^{A21} des(B30) human insulin,
 N^{εB29}-decanoyl Gly^{A21} des(B30) human insulin,
 N^{εB29}-dodecanoyl Gly^{A21} des(B30) human insulin,
 N^{εB29}-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-tridecanoyl Ala^{A21} des(B30) human insulin,
 N^{εB29}-tetradecanoyl Ala^{A21} des(B30) human insulin,
 N^{εB29}-decanoyl Ala^{A21} des(B30) human insulin,
 N^{εB29}-dodecanoyl Ala^{A21} des(B30) human insulin,
 N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
 N^{εB29}-tridecanoyl Gln^{B3} des(B30) human insulin,
 N^{εB29}-tetradecanoyl Gln^{B3} des(B30) human insulin,
 N^{εB29}-decanoyl Gln^{B3} des(B30) human insulin,
 N^{εB29}-dodecanoyl Gln^{B3} des(B30) human insulin,
 N^{εB29}-tridecanoyl Gly^{A21} human insulin,

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$N^{\epsilon B29}$ -tetradecanoyl Gly^{A21} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gly^{A21} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gly^{A21} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gly^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gly^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gly^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gly^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Ala^{A21} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} human insulin,
 $N^{\epsilon B29}$ -decanoyl Ala^{A21} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Ala^{A21} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -decanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gln^{B3} Glu^{B30} human insulin and
 $N^{\epsilon B29}$ -dodecanoyl Gln^{B3} Glu^{B30} human insulin.

Examples of preferred human insulin derivatives according to the present invention in which two Zn^{2+} ions are bound per insulin hexamer are the following:

bound per insulin hexamer are the following:

$$\begin{aligned}
 &(\text{N}^{\text{E}29}\text{-tridecanoyl des(B30) human insulin})_6, 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-tetradecanoyl des(B30) human insulin})_6, 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-decanoyl des(B30) human insulin})_6, 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-dodecanoyl des(B30) human insulin})_6, 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-tridecanoyl Gly}^{\text{A}21}\text{ des(B30) human insulin})_6, 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-tetradecanoyl Gly}^{\text{A}21}\text{ des(B30) human insulin})_6, \\
 &\quad 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-decanoyl Gly}^{\text{A}21}\text{ des(B30) human insulin})_6, 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-dodecanoyl Gly}^{\text{A}21}\text{ des(B30) human insulin})_6, \\
 &\quad 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-tridecanoyl Gly}^{\text{A}21}\text{ Gln}^{\text{B}3}\text{ des(B30) human insulin})_6, \\
 &\quad 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-tetradecanoyl Gly}^{\text{A}21}\text{ Gln}^{\text{B}3}\text{ des(B30) human insulin})_6, \\
 &\quad 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-decanoyl Gly}^{\text{A}21}\text{ Gln}^{\text{B}3}\text{ des(B30) human insulin})_6, \\
 &\quad 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-dodecanoyl Gly}^{\text{A}21}\text{ Gln}^{\text{B}3}\text{ des(B30) human insulin})_6, \\
 &\quad 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-tridecanoyl Ala}^{\text{A}21}\text{ des(B30) human insulin})_6, 2\text{Zn}^{2+}, \\
 &(\text{N}^{\text{E}29}\text{-tetradecanoyl Ala}^{\text{A}21}\text{ des(B30) human insulin})_6, \\
 &\quad 2\text{Zn}^{2+}.
 \end{aligned}$$

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(N^{εB29}-decanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆,
2Zn²⁺,
5 (N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)
6, 2Zn²⁺,
(N^{εB29}-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆,
2Zn²⁺,
(N^{εB29}-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆,
2Zn²⁺,
10 (N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆,
(N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆,
2Zn²⁺,
(N^{εB29}-decanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
15 (N^{εB29}-dodecanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl human insulin)₆, 2Zn²⁺,
(N^{εB29}-tetradecanoyl human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl human insulin)₆, 2Zn²⁺,
20 (N^{εB29}-tridecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tetradecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
25 (N^{εB29}-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tetradecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
30 (N^{εB29}-decanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
35 (N^{εB29}-dodecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tetradecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
40 (N^{εB29}-tridecanoyl Gln^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tetradecanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
45 (N^{εB29}-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺,
50 (N^{εB29}-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺,
(N^{εB29}-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺,
(N^{εB29}-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺,
55 (N^{εB29}-tridecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-decanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
(N^{εB29}-dodecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
60 (N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺,
(N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺,
(N^{εB29}-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺,
65 (N^{εB29}-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
2Zn²⁺.

(N^{εB29}-decanoyl human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl Gly^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl Ala^{A21} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl Gln^{B3} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-tridecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-dodecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆,
 4Zn²⁺,
 (N^{εB29}-tridecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^{εB29}-decanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺ and
 (N^{εB29}-dodecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated with reference to the appended drawings wherein

FIG. 1 shows the construction of the plasmid pEA5.3.2;

FIG. 2 shows the construction of the plasmid pEA108; and

FIG. 3 shows the construction of the plasmid pEA113.

DETAILED DESCRIPTION OF THE INVENTION

Terminology

The three letter codes and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. 243, p. 3558 (1968).

In the DNA sequences, A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms are used: DMSO for dimethyl sulphoxide, DMF for dimethylformamide, Boc for tert-butoxycarbonyl, RP-HPLC for reversed phase high performance liquid chromatography, X-OSu is an N-hydroxysuccinimide ester, X is an acyl group, and TFA for trifluoroacetic acid.

Preparation of Lipophilic Insulin Derivatives

The insulin derivatives according to the present invention can be prepared i.a. as described in the following:

1. Insulin Derivatives Featuring in Position B30 an Amino Acid Residue Which can be Coded for by the Genetic Code e.g. Threonine (Human Insulin) or Alanine (Porcine Insulin)

1.1 Starting from Human Insulin

Human insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc human insulin, i.e., human insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ε-amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, NE^{B29}-X human insulin, is isolated.

1.2 Starting from a Single Chain Insulin Precursor

A single chain insulin precursor, extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and in which the bridge from B30 to A1 is an arginine residue, i.e. a compound of the general formula Ext-Arg-B(1-30)-Arg-A(1-21), can be used as starting material. Acylation of this starting material with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ε-amino group of Lys^{B29} and in the N-terminal amino group of the precursor. On treating this acylated precursor of the formula (N^{εB29}-X),X-Ext-Arg-B(1-30)-Arg-A(1-21) with trypsin in a mixture of water and a suitable water-miscible organic solvent, e.g. DMF, DMSO or a lower alcohol, an intermediate of the formula (N^{εB29}-X),Arg^{B31} insulin is obtained. Treating this intermediate with carboxypeptidase B yields the desired product, (N^{εB29}-X) insulin.

2. Insulin Derivatives with no Amino Acid Residue in Position B30, i.e. des(B30) Insulins

2.1 Starting from Human Insulin or Porcine Insulin

On treatment with carboxypeptidase A in ammonium buffer, human insulin and porcine insulin both yield des(B30) insulin. After an optional purification, the des(B30) insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc des(B30) insulin, i.e., des(B30) insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ε-amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, (N^{εB29}-X) des(B30) insulin, is isolated.

2.2 Starting from a Single Chain Human Insulin Precursor

A single chain human insulin precursor, which is extended in position B1 with an extension (Ext) which is connected to

B1 via an arginine residue and which has a bridge from B30 to A1 can be a useful starting material. Preferably, the bridge is a peptide of the formula Y_n -Arg, where Y is a codable amino acid except lysine and arginine, and n is zero or an integer between 1 and 35. When n>1, the Y's may designate different amino acids. Preferred examples of the bridge from B30 to A1 are: AlaAlaArg, SerArg, SerAspAspAlaArg and Arg (European Patent No. 163529). Treatment of such a precursor of the general formula Ext-Arg-B(1-30)- Y_n -Arg-A(1-21) with a lysyl endopeptidase, e.g. *Achromobacter lyticus* protease, yields Ext-Arg-B(1-29) Thr- Y_n -Arg-A(1-21) des(B30) insulin. Acylation of this intermediate with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ -amino group of Lys^{B29}, and in the N-terminal amino group of the A-chain and the B-chain to give (N^{B29}-X) X-Ext-Arg-B(1-29) X-Thr- Y_n -Arg-A(1-21) des(B30) insulin. This intermediate on treatment with trypsin in mixture of water and a suitable organic solvent, e.g. DMF, DMSO or a lower alcohol, gives the desired derivative, (N^{B29}-X) des (B30) human insulin.

Data on N^{B29} Modified Insulins

Certain experimental data on N^{B29} modified insulins are given in Table 1.

(prolongation), see p. 211 in Markussen et al., Protein Engineering 1 (1987) 205-213. The formula has been scaled to render a value of 100 with bovine ultralente insulin and a value of 0 with Actrapid® insulin (Novo Nordisk A/S, 2880 Bagsvaerd, Denmark).

The insulin derivatives listed in Table 1 were administered in solutions containing 3 Zn²⁺ per insulin hexamer, except those specifically indicated to be Zn-free.

For the very protracted analogues the rabbit model is inadequate because the decrease in blood glucose from initial is too small to estimate the index of protraction. The prolongation of such analogues is better characterized by the disappearance rate in pigs. T_{50%} is the time when 50% of the A14 Tyr(¹²⁵I) analogue has disappeared from the site of injection as measured with an external γ -counter (Ribel, U et al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. serrano-Rios and P. J. Lefebvre (Eds): Diabetes 1985; Proceedings of the 12th Congress of the International Diabetes Federation, Madrid, Spain, 1985 (Excerpta Medica, Amsterdam, (1986) 891-96).

In Table 2 are given the T_{50%} values of a series of very protracted insulin analogues. The analogues were administered in solutions containing 3 Zn²⁺ per insulin hexamer.

TABLE 1

Insulin Derivative *)	Relative Lipophilicity	Blood glucose, % of initial				Index of protraction
		1 h	2 h	4 h	6 h	
N ^{B29} -benzoyl insulin	1.14					
N ^{B29} -phenylacetyl insulin (Zn-free)	1.28	55.4	58.9	88.8	90.1	10
N ^{B29} -cyclohexylacetyl insulin	1.90	53.1	49.6	66.9	81.1	28
N ^{B29} -cyclohexylpropionyl insulin	3.29	55.5	47.6	61.5	73.0	39
N ^{B29} -cyclohexylvaleroyl insulin	9.87	65.0	58.3	65.7	71.0	49
N ^{B29} -octanoyl insulin	3.97	57.1	54.8	69.0	78.9	33
N ^{B29} -decanoyl, des-(B30) insulin	11.0	74.3	65.0	60.9	64.1	65
N ^{B29} -decanoyl insulin	12.3	73.3	59.4	64.9	68.0	60
N ^{B29} -undecanoyl, des-(B30) insulin	19.7	88.1	80.0	72.1	72.1	80
N ^{B29} -lauroyl, des-(B30) insulin	37.0	91.4	90.0	84.2	83.9	78
N ^{B29} -myristoyl insulin	113	98.5	92.0	83.9	84.5	97
N ^{B29} -choleoyl insulin	7.64	58.2	53.2	69.0	88.5	20
N ^{B29} -7-deoxycholeoyl insulin (Zn-free)	24.4	76.5	65.2	77.4	87.4	35
N ^{B29} -lithocholoyl insulin (Zn-free)	51.6	98.3	92.3	100.5	93.4	115
N ^{B29} -4-benzoyl-phenylalanyl insulin	2.51	53.9	58.7	74.4	89.0	14
N ^{B29} -3,5-diiodotyrosyl insulin	1.07	53.9	48.3	60.8	82.1	27
N ^{B29} -L-thyroxylin insulin	8.00					

*) 3 Zn²⁺/insulin hexamer except where otherwise indicated.

The lipophilicity of an insulin derivative relative to human insulin, k'_{rel} , was measured on a LiChrosorb RP18 (5 μ m, 250x4 mm) HPLC column by isocratic elution at 40° C. using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10% acetonitrile, and B) 50% acetonitrile in water as eluents. The elution was monitored by following the UV absorption of the eluate at 214 nm. Void time, t_0 , was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, t_{human} , was adjusted to at least 2 t_0 by varying the ratio between the A and B solutions. $k'_{rel} = (t_{derivative} - t_0) / (t_{human} - t_0)$.

The degree of prolongation of the blood glucose lowering effect was studied in rabbits. Each insulin derivative was tested by subcutaneous injection of 12 nmol thereof in each of six rabbits in the single day retardation test. Blood sampling for glucose analysis was performed before injection and at 1, 2, 4 and 6 hours after injection. The glucose values found are expressed as percent of initial values. The Index of Protraction, which was calculated from the blood glucose values, is the scaled Index of Protraction

TABLE 2

Derivative of Human Insulin 600 μ M, 3 Zn ²⁺ /hexamer, phenol 0.3%, glycerol 1.6%, pH 7.5	Relative hydrophobicity k'_{rel}	Subcutaneous disappearance in pigs T _{50%} , hours
N ^{B29} -decanoyl des(B30) insulin	11.0	5.6
N ^{B29} -undecanoyl des(B30) insulin	19.7	6.9
N ^{B29} -lauroyl des(B30) insulin	37	10.1
N ^{B29} -tridecanoyl des(B30) insulin	65	12.9
N ^{B29} -myristoyl des(B30) insulin	113	13.8
N ^{B29} -palmitoyl des(B30) insulin	346	12.4
N ^{B29} -2-succinyl-amido myristic acid insulin	10.5	13.6
N ^{B29} -myristoyl insulin	113	11.9
N ^{B29} -2-succinyl-amido palmitic acid insulin	420	20.1
N ^{B29} -myristoyl- α -glutamyl des(B30) insulin	23.7	8.8
N ^{B29} -myristoyl- α -glutamyl-glycyl des(B30) insulin	20.0	11.9

TABLE 2-continued

Derivative of Human Insulin 600 μ M, 3 Zn^{2+} /hexamer, phenol 0.3%, glycerol 1.6%, pII 7.5	Relative hydrophobicity k'_{rel}	Subcutaneous disappearance in pigs $T_{50\%}$, hours
$N^{\epsilon B29}$ -lithocholoyl- α -glutamyl des(B30) insulin	12.5	14.3
Human NPH		10

Solubility

The solubility of all the $N^{\epsilon B29}$ modified insulins mentioned in Table 1, which contain 3 Zn^{2+} ions per insulin hexamer, exceeds 600 nmol/ml in a neutral (pH 7.5), aqueous, pharmaceutical formulation which further comprises 0.3% phenol as preservative, and 1.6% glycerol to achieve isotonicity. 600 nmol/ml is the concentration of human insulin found in the 100 IU/ml compositions usually employed in the clinic.

The ϵ -B29 amino group can be a component of an amide bond, a sulphonamide bond, a carbamide, a thiocarbamide, or a carbamate. The lipophilic substituent carried by the ϵ -B29 amino group can also be an alkyl group.

Pharmaceutical compositions containing a human insulin derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the human insulin derivative in the form of a nasal spray.

The injectable human insulin compositions of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

Thus, according to one procedure, the human insulin derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted—if necessary—using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride, mannitol and glycerol.

Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

A composition for nasal administration of an insulin derivative according to the present invention may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific human insulin derivative employed,

the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Where expedient, the human insulin derivatives of this invention may be used in mixture with other types of insulin, e.g. human insulin or porcine insulin or insulin analogues with a more rapid onset of action. Examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826 (Novo Nordisk A/S), EP 375437 (Novo Nordisk A/S) and EP 383472 (Eli Lilly & Co.).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Plasmids and DNA Material

All expression plasmids are of the cPOT type. Such plasmids are described in EP patent application No. 171 142 and are characterized in containing the *Schizosaccharomyces pombe* triose phosphate isomerase gene (POT) for the purpose of plasmid selection and stabilization. A plasmid containing the POT-gene is available from a deposited *E. coli* strain (ATCC 39685). The plasmids furthermore contain the *S. cerevisiae* triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pMT742 (Egel-Mitani, M. et al., *Gene* 73 (1988) 113–120) (see FIG. 1) except for the region defined by the EcoRI-XbaI restriction sites encompassing the coding region for signal/leader/product.

Synthetic DNA fragments were synthesized on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S. L. and Caruthers, M. H., *Tetrahedron Letters* 22 (1981) 1859–1869).

All other methods and materials used are common state of the art knowledge (see, e.g. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989).

Analytical

Molecular masses of the insulins prepared were obtained by MS (mass spectroscopy), either by PDMS (plasma desorption mass spectrometry) using a Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden) or by ESMS (electrospray mass spectrometry) using an API III Biomolecular Mass Analyzer (Perkin-Elmer Sciex Instruments, Thornhill, Canada).

Example 1

Synthesis of Ala^{A21} Asp^{B3} Human Insulin Precursor from Yeast Strain yEA002 Using the LaC212spX3 Signal/Leader

The following oligonucleotides were synthesized:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCACTGGTTGAA
(SEQ ID NO:3)

GCTTTGTACTTGGTTTGTGGTGAAAGAGTTTCTTCTACACTCCAAAGTCTGA

CGACGCT-3' (Asp^{B3})

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTCCAATTGGTACAAAGAACAG
(SEQ ID NO:4)

ATAGAAGTACAACATTGTTCAACGATACCCCTTAGCGTCGTACAGCTTTGG-3'
(Ala^{A21})

#126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3}) (SEQ ID NO:5)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avenue, Conn. 06859, U.S.A.) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with 100 μ l of mineral oil (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

2.5 μ l of oligonucleotide #98 (2.5 pmol)

2.5 μ l of oligonucleotide #128 (2.5 pmol)

10 μ l of 10 \times PCR buffer

16 μ l of dNTP mix

0.5 μ l of Taq enzyme

58.5 μ l of water

One cycle was performed: 94° C. for 45 sec., 49° C. for 1 min, 72° C. for 2 min.

Subsequently, 5 μ l of oligonucleotides #16 and #126 was added and 15 cycles were performed: 94° C. for 45 sec., 45° C. for 1 min, 72° C. for 1.5 min. The PCR mixture was loaded onto a 2.5% agarose gel and subjected to electrophoresis using standard techniques (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989). The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean Kit (Bio 101 Inc., PO BOX 2284, La Jolla, Calif., 92038, U.S.A.) according to the manufacturer's instructions. The purified PCR DNA fragment was dissolved in 10 μ l of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques, run on a 2.5% agarose gel and purified using the Gene Clean Kit as described.

The plasmid pAK188 consists of a DNA sequence of 412 bp composed of a EcoRI/NcoI fragment encoding the synthetic yeast signal/leader gene LaC212sp3 (described in Example 3 of WO 89/02463) followed by a synthetic NcoI/XbaI fragment encoding the insulin precursor M15, which has a SerAspAspAlaLys bridge connecting the B29 and the A1 amino acid residues (see SEQ ID NOS. 14, 15 and 16), inserted into the EcoRI/XbaI fragment of the vector (phagemid) pBLUESCRIPT II sk(±) (Stratagene, U.S.A.). The plasmid pAK188 is shown in FIG. 1.

The plasmid pAK188 was also cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3139 bp isolated. The two DNA fragments were ligated together using T4 DNA ligase and standard conditions (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989). The ligation mixture was transformed into a competent *E. coli* strain (R⁻, M⁺) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using standard DNA miniprep technique (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989), checked with appropriate restrictions endonucleases i.e. EcoRI, XbaI,

NcoI and HpaI. The selected plasmid was shown by DNA sequencing analyses (Sequenase, U.S. Biochemical Corp.) to contain the correct sequence for the Ala^{A21}, Asp^{B3} human insulin precursor and named pEA5.3.

The plasmid pKFN1627 is an *E. coli*-*S. cerevisiae* shuttle vector, identical to plasmid pKFN1003 described in EP patent No. 375718, except for a short DNA sequence upstream from the unique XbaI site. In pKFN1003, this sequence is a 178 bp fragment encoding a synthetic aprotinin gene fused in-frame to the yeast mating factor alpha 1 signal-leader sequence. In pKFN1627, the corresponding 184 bp sequence encodes the insulin precursor M15 (Glu^{B1}, Glu^{B28}) (i.e. B(1-29, Glu^{B1}, Glu^{B28})-SerAspAspAlaLys-A (1-21) fused in-frame to the mating factor alpha 1 sequence (see SEQ ID NOS. 17, 18 and 19). The vector pKFN1627 is shown in FIG. 1.

pEA5.3 was cut with the restriction endonucleases EcoRI and XbaI and the resulting DNA fragment of 412 bp was isolated. The yeast expression vector pKFN1627 was cut with the restriction endonucleases NcoI and XbaI and with NcoI and EcoRI and the DNA fragment of 9273 bp was isolated from the first digestion and the DNA fragment of 1644 bp was isolated from the second. The 412 bp EcoRI/XbaI fragment was then ligated to the two other fragments, that is the 9273 bp NcoI/XbaI fragment and the 1644 bp NcoI/EcoRI fragment using standard techniques.

The ligation mixture was transformed into *E. coli* as described above. Plasmid from the resulting *E. coli* was isolated using standard techniques, and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HpaI. The selected plasmid was shown by DNA sequence analysis (using the Sequenase kit as described by the manufacturer, U.S. Biochemical) to contain the correct sequence for the Ala^{A21} Asp^{B3} human insulin precursor DNA and to be inserted after the DNA encoding the LaC212sp3 signal/leader DNA. The plasmid was named pEA5.3.2 and is shown in FIG. 1. The DNA sequence encoding the LaC212sp3 signal/leader/Ala^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 20, 21 and 22. The plasmid pEA5.3.2 was transformed into *S. cerevisiae* strain MT663 as described in European patent application having the publication No. 214826 and the resulting strain was named yEA002.

Example 2

Synthesis of Ala^{A21} Thr^{B3} Human Insulin Precursor
from Yeast Strain yEA005 Using the LaC212sp3
Signal/Leader

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTTCGTTACTCAACACTTGTGCGGTTCTCACTT (SEQ ID NO:7)

GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAGAGGTTTCTTCTACA

CTCCAAAGTCTGACGACGCT-3' (Thr^{B3})

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTCCAATTGGTACAAA (SEQ ID NO:4)

GAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCG

TCAGACTTTGG-3' (Ala^{A21})

#15 5'-GTCGCCATGGCTAAGAGATTTCGTTA-3' (Thr^{B3}) (SEQ ID NO:8)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Ala^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212sp3 signal/leader/Ala^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 23, 24 and 25. The plasmid pEA8.1.1 was shown to contain the desired sequence, transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA005.

Example 3

Synthesis of Gly^{A21} Asp^{B3} Human Insulin Precursor from Yeast Strain yEA007 Using the LaC212sp3 Signal/Leader

The following oligonucleotides were synthesized:

#98 5'-TGGCTAAGAGATTTCGTTGACCAACACTTGTGCGGTTCTCACTT (SEQ ID NO:3)

GTGAAGCTTTGTACTTGGTTTGTGGTGAAGAGGTTTCTTCT

ACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3})

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTCCAATTGGTACAA (SEQ ID NO:9)

AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT

-continued

TAGCGTCGTCAGACTTTGG-3' (Gly^{A21})

#126 5'-GTCGCCATGGCTAAGAGATTTCGTTG-3' (Asp^{B3}) (SEQ ID NO:5)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Gly^{A21} Asp^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212sp3 signal/leader/Gly^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 26, 27 and 28. The plasmid pEA1.5.6 was shown to contain the desired sequence, transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA007.

Example 4

Synthesis of Gly^{A21} Thr^{B3} Human Insulin Precursor from Yeast Strain yEA006 Using the LaC212sp3 Signal/Leader

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTTCGTTACTCAACACTTGTGCGGTTCTCACTTGGTTGAAG (SEQ ID NO:7)

CTTGTACTTGGTTTGTGGTGAAGAGGTTTCTTCTACACTCCAAAGTCTGACG

ACGCT-3' (Thr^{B3})

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTCCAATTGGTACAAAGAACAG (SEQ ID NO:9)

ATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCGTCAGACTTTGG-3' (Gly^{A21})

#15 5'-GTCGCCATGGCTAAGAGATTTCGTTA-3' (Thr^{B3}) (SEQ ID NO:8)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

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The DNA encoding Gly^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Gly^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 29, 30 and 31. The plasmid pEA4.4.11 was shown to contain the desired DNA sequence, transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA006.

Example 5

Synthesis of Arg^{B-1} Arg^{B31} Single Chain Human Insulin Precursor Having an N-Terminal Extension (GluGluAlaGluAlaGluAlaArg) from Yeast Strain yEA113 Using the Alpha Factor Leader

A) The following oligonucleotides were synthesized:

#220 5'-ACGTACGTTCTAGAGCCTGCGGGCTGC-3' (SEQ ID NO:10)

#263 5'-CACTTGCTTGAAGCTTTGTACTTGGTTTGTGGTGAAGAGGTTTC (SEQ ID NO:11)

TTCTACACTCCAAGACTAGAGGTATCGTTGAA-3'

#307 5'-GCTAACGTGCGCCATGGCTAAGAGAGAAGAAGCTGAAGCTGAAGCT (SEQ ID NO:12)

AGATTTCGTTAACCAACAC-3'

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avenue, Conn. 06859, U.S.A.) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with 100 μ l of mineral oil (Sigma Chemical Co., St. Louis, Mo., U.S.A.). The plasmid pAK220 (which is identical to pAK188) consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the insulin precursor M15 (see SEQ ID NOS. 14, 15 and 16) inserted into the vector (phagemid) pBLUE-SCRIPT II sk(±) (Stratagene, U.S.A.).

5 μ l of oligonucleotide #220 (100 pmol)

5 μ l of oligonucleotide #263 (100 pmol)

10 μ l of 10 \times PCR buffer

16 μ l of dNTP mix

0.5 μ l of Taq enzyme

0.5 μ l of pAK220 plasmid (identical to pAK188) as template (0.2 μ g of DNA)

63 μ l of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95° C.; 1 minute at 40° C.; and 2 minutes at 72° C. The PCR mixture was then loaded onto a 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, Calif., 92038, U.S.A.) according to the manufacturer's instructions. The purified PCR DNA fragment was dissolved in 10 μ l of water and restriction endonuclease buffer and cut with the restriction endonucleases HindIII and XbaI according to standard techniques. The HindIII/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK406 consists of a DNA sequence of 520 bp comprising an EcoRI/HindIII fragment derived from pMT636 (described in WO 90/10075) encoding the yeast alpha factor leader and part of the insulin precursor ligated

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to the HindIII/XbaI fragment from pAK188 encoding the rest of the insulin precursor M15 (see SEQ ID NOS. 32, 33 and 34) inserted into the vector cPOT. The vector pAK406 is shown in FIG. 2.

The plasmid pAK233 consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the gene for the insulin precursor B(1-29)-GluLysArg-A(1-21) (A21-Gly) (see SEQ ID NOS. 35, 36 and 37) inserted into the vector cPOT. The plasmid pAK233 is shown in FIG. 2.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 9273 bp isolated. The plasmid pAK406 was cut with the restriction endonucleases NcoI and HindIII and the vector fragment of 2012 bp isolated. These two DNA fragments were ligated together with the HindIII/XbaI PCR fragment using T4 DNA ligase and standard conditions. The ligation mixture was then transformed into a competent *E. coli* strain (R-,

M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA and to be inserted after the DNA encoding the *S. cerevisiae* alpha factor DNA. The plasmid was named pEA108 and is shown in FIG. 2. The DNA sequence encoding the alpha factor leader/Arg^{B31} single chain human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 38, 39 and 40. The plasmid pEA108 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA108.

B) The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avenue, Conn. 06859, U.S.A.) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with 100 μ l of mineral oil (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

5 μ l of oligonucleotide #220 (100 pmol)

5 μ l of oligonucleotide #307 (100 pmol)

10 μ l of 10 \times PCR buffer

16 μ l of dNTP mix

0.5 μ l of Taq enzyme

0.2 μ l of pEA108 plasmid as template (0.1 μ g DNA)

63 μ l of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95° C.; 1 minute at 40° C.; and 2 minutes at 72° C. The PCR mixture was then loaded onto a 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, Calif., 92038, U.S.A.) according to the manufacturer's instructions. The purified PCR DNA fragment was dissolved in 10 μ l of water and restric-

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tion endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques. The NcoI/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK401 consists of a DNA sequence of 523 bp composed of an EcoRI/NcoI fragment derived from pMT636 (described in WO 90/10075) (constructed by by introducing a NcoI site in the 3'-end of the alpha leader by site directed mutagenesis) encoding the alpha factor leader followed by a NcoI/XbaI fragment from pAK188 encoding the insulin precursor M15 (see SEQ ID NOS. 41, 42 and 43) inserted into the vector (phagemid) pBLUESCRIPT II sk(±) (Stratagene, U.S.A.). The plasmid pAK401 is shown in FIG. 3.

The plasmid pAK401 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3254 bp isolated and ligated together with the NcoI/XbaI PCR fragment. The ligation mixture was then transformed into a competent *E. coli* strain and plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI. The selected plasmid, named p113A (shown in FIG. 3), was cut with EcoRI and XbaI and the fragment of 535 bp isolated.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI, and with EcoRI/NcoI and the fragments of 9273 and 1644 bp isolated. These two DNA fragments were ligated together with the EcoRI/XbaI fragment from p113A using T4 DNA ligase and standard conditions. The ligation mixture was then transformed into a competent *E. coli* strain (R⁻, M⁺) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA with the N-terminal extension GluGluAlaGluAlaGluAlaArg and to be inserted after the DNA encoding the *S. cerevisiae* alpha factor DNA. The plasmid was named pEA113 and is shown in FIG. 3. The DNA sequence encoding the alpha factor leader/Arg^{B-1} ArgB31 single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) and the amino acid sequence thereof are SEQ ID NOS. 44, 45 and 46. The plasmid pEA113 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA113.

Example 6

Synthesis of Arg^{B-1} Arg^{B31} Single Chain Human Insulin Precursor Having an N-Terminal Extension (GluGluAlaGluAlaGluAlaArg) from Yeast Strain yEA136 Using the Alpha Factor Leader

The following oligonucleotide was synthesized:

#389 5'-GCTAACGTCGCCATGGCTAAGAGAGAAG-AAGCTGAAGCGAAGCTGAAAGATTCGTAAACCAACAC-3' (SEQ ID NO:13)

The following PCR was performed using the Gene Amp PCR reagent kit

5 µl of oligonucleotide #220 (100 pmol)

5 µl of oligonucleotide #389 (100 pmol)

10 µl of 10× PCR buffer

16 µl of dNTP mix

0.5 µl of Taq enzyme

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0.2 µl of pEA113 plasmid as template (0.5 ug DNA)
63 µl of water

A total of 12 cycles were performed, each cycle comprising 1 minute at 95° C.; 1 minute at 37° C.; and 2 minutes at 72° C.

The DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) was constructed in the same manner as described for the DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) in Example 5. The plasmid was named pEA136. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) and the amino acid sequence thereof are SEQ ID NOS. 47, 48 and 49. The plasmid pEA136 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA136.

Example 7

Synthesis of (A1,B1)-diBoc Human Insulin

5 g of zinc-free human insulin was dissolved in 41.3 ml of DMSO. To the solution was added 3.090 ml of acetic acid. The reaction was conducted at room temperature and initiated by addition of 565 mg of di-tert-butyl pyrocarbonate dissolved in 5.650 ml of DMSO. The reaction was allowed to proceed for 5½ hour and then stopped by addition of 250 µl of ethanolamine. The product was precipitated by addition of 1500 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. A yield of 6.85 g material was obtained.

(A1,B1)-diBoc insulin was purified by reversed phase HPLC as follows: The crude product was dissolved in 100 ml of 25% ethanol in water, adjusted to pH 3.0 with HCl and applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 µm, pore size 100 Å) and equilibrated with elution buffer. The elution was performed using mixtures of ethanol and 1 mM aqueous HCl, 0.3 M KCl at a flow of 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 45%. The appropriate fraction was diluted to 20% ethanol and precipitated at pH 4.8. The precipitated material was isolated by centrifugation and dried in vacuum. Thus 1.701 g of (A1,B1)-diBoc human insulin was obtained at a purity of 94.5%.

Example 8

Synthesis of (N^{B29}-benzoyl Human Insulin)₆, 3Zn²⁺

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 µl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15° C. and initiated by addition of 14.6 mg of benzoic acid N-hydroxysuccinimide ester dissolved in 132 µl DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 343 mg of material was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum.

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N^εB²⁹-benzoyl human insulin was purified by reversed phase HPLC as described in Example 7. A yield of 230 mg was obtained. Recrystallization from 15% aqueous ethanol containing 6 mM Zn²⁺ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 190 mg. Molecular mass, found by MS: 5911, theory: 5911.

Example 9

Synthesis of (N^εB²⁹-lithocholoyl Human Insulin)₆, 3Zn²⁺

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15° C. and initiated by addition of 31.94 mg of lithocholic acid N-hydroxysuccinimide ester dissolved in 300 μl of DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 331 mg of material was obtained.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield was 376 mg.

B29-lithocholoyl insulin was purified by reversed phase HPLC as described in Example 7. A final yield of 67 mg was obtained at a purity of 94%. Recrystallization from 15% aqueous ethanol containing 6 mM Zn²⁺ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 49 mg.

Molecular mass, found by MS: 6160, theory: 6166.

Example 10

Synthesis of (N^εB²⁹-decanoyl Human Insulin)₆, 3Zn²⁺

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15° C. and initiated by addition of 18.0 mg of decanoic acid N-hydroxysuccinimide ester dissolved in 132 μl of DMF. The reaction was stopped after 60 minutes and the product precipitated by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 420 mg of intermediate product was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and the product was then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield of crude product was 420 mg.

The crude product was purified by reversed phase HPLC as described in Example 7. A final yield of 254 mg of the title product was obtained. The purity was 96.1%. Recrystallization from 15% aqueous ethanol containing 6 mM Zn²⁺ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 217 mg.

Molecular mass, found by MS: 5962, theory: 5962.

Example 11

Synthesis of des(B30) Human Insulin

Synthesis of des(B30) human insulin was carried out as described by Markussen (Methods in diabetes research, Vol.

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I, Laboratory methods, part B, 404-410. Ed: J. Larner and S. Phol, John Wiley & Sons, 1984). 5 g of human insulin was dissolved in 500 ml of water while the pH value of the solution was kept at 2.6 by addition of 0.5 M sulphuric acid. Subsequently, the insulin was salted out by addition of 100 g of ammonium sulphate and the precipitate was isolated by centrifugation. The pellet was dissolved in 800 ml of 0.1 M ammonium hydrogen carbonate and the pH value of the solution was adjusted to 8.4 with 1 M ammonia.

50 mg of bovine carboxypeptidase A was suspended in 25 ml of water and isolated by centrifugation. The crystals were suspended in 25 ml of water and 1 M ammonia was added until a clear solution was obtained at a final pH of 10. The carboxypeptidase solution was added to the insulin solution and the reaction was allowed to proceed for 24 hours. A few drops of toluene were added to act as preservative during the reaction.

After 24 hours the des(B30) human insulin was crystallized by successive addition of 80 g of sodium chloride while the solution was stirred. The pH value was then adjusted to 8.3 and the crystallization was allowed to proceed for 20 hours with gentle stirring. The crystals were isolated on a 1.2 μl filter, washed with 250 ml of ice cold 2-propanol and finally dried in vacuum.

Example 12

Synthesis of (A1,B1)-diBoc des(B30) Human Insulin

The title compound was synthesized by a method similar to that described in Example 7, using des(B30) porcine insulin as the starting material. The crude product was precipitated by acetone and dried in vacuum. The (A1,B1)-diBoc des(B30) human insulin was purified by reversed phase HPLC as described in Example 7.

Example 13

Synthesis of N^εB²⁹-decanoyl des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was used as starting material for the synthesis of N^εB²⁹-decanoyl des(B30) human insulin, following the procedure described in Example 10. The crude product was precipitated by acetone, dried in vacuum and deprotected using TFA. The resulting product was precipitated by acetone and dried in vacuum. N^εB²⁹-decanoyl des(B30) human insulin was then purified by reversed phase HPLC as described in Example 10.

Molecular mass, found by MS: 5856, theory: 5861.

Example 14

Synthesis of N^εB²⁹-dodecanoyl des(B30) Human Insulin

a. Immobilization of *A. lyticus* Protease

13 mg of *A. lyticus* protease, dissolved in 5 ml of aqueous 0.2 M NaHCO₃ buffer, pH 9.4, was mixed with 4 ml of settled MiniLeak® Medium gel, which had been washed with the same buffer (MiniLeak is a divinylsulfone activated Sepharose CL 6B, obtained from KemEnTec, Copenhagen). The gel was kept in suspension by gentle stirring for 24 hours at room temperature. Then, the gel was isolated by filtration, washed with water, and suspended in 20 ml of 1 M ethanolamine buffer, pH 9.4, and kept in suspension for 24 hours at room temperature. Finally, the gel was washed

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with water followed by 0.1 M acetic acid and stored at 4° C. The enzyme activity in the filtrate was 13% of that in the initial solution, indicating a yield in the immobilization reaction of about 87%.

b. Immobilization of Porcine Trypsin

Porcine trypsin was immobilized to MiniLeak® Low to a degree of substitution of 1 mg per ml of gel, using the conditions described above for immobilization of *A. lyticus*.
c. Synthesis of Glu(GluAla)₃Arg-B(1-29), ThrArg-A(1-21) Insulin Using Immobilized *A. lyticus* Protease

To 200 mg of Glu(GluAla)₃Arg-B(1-29)-ThrArg-A(1-21) single-chain human insulin precursor, dissolved in 20 ml of 0.1 M NaHCO₃ buffer, pH 9.0, was added 4 ml of the gel carrying the immobilized *A. lyticus* protease. After the gel had been kept in suspension in the reaction mixture for 6 hours at room temperature the hydrolysis was complete, rendering Glu(GluAla)₃Arg-B(1-29), ThrArg-A(1-21) human insulin (the reaction was followed by reversed phase HPLC). After the hydrolysis, the gel was removed by filtration. To the filtrate was added 5 ml of ethanol and 15 μ l of 1 M ZnCl₂ and the pH was adjusted to 5.0 using HCl. The precipitation of the product was completed on standing overnight at 4° C. with gentle stirring. The product was isolated by centrifugation. After one washing with 1 ml of ice cold 20% ethanol and drying in vacuo the yield was 190 mg.

d. Synthesis of N^{αA1},N^{αB1},N^{εB29}-tridecanoyl Glu(GluAla)₃Arg-B(1-29), ThrArg-A(1-21) Human Insulin Using Dodecanoic Acid N-hydroxysuccinimide Ester

190 mg (30 μ mol) of Glu(GluAla)₃Arg-B(1-29),ThrArg-A(1-21) insulin was dissolved in 1 ml of DMSO and 1.05 ml of a 0.572 M solution of N,N-diisopropylethylamine in DMF. The solution was cooled to 15° C. and 36 mg (120 μ mol) of dodecanoic acid N-hydroxysuccinimide ester dissolved in 0.6 ml of DMSO was added. The reaction was completed within 24 hours. The lipophilic title compound was not isolated.

e. Synthesis of N^{εB29}-dodecanoyl des(B30) Insulin

The product from the previous step, d., contained in approximately 2.65 ml of DMSO/DMF/N,N-diisopropylethylamine was diluted with 10.6 ml of a 50 mM glycine buffer comprising 20% ethanol and the pH adjusted to 10 with NaOH. After standing for 1 hour at room temperature 1 ml of MiniLeak gel, carrying 1 mg of immobilized trypsin per ml of gel, was added. The reaction mixture was stirred gently for 48 hours at room temperature. In order to isolate the desired product, the reaction mixture was applied to a reversed phase HPLC column (5 cm in diameter, 30 cm high), packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μ m, pore size 100 Å). For the elution was used 20 mM Tris/HCl buffers, adjusted to pH 7.7 and comprising an increasing concentration of ethanol, from 40% to 44% (v/v), at a rate of 2000 ml/h. The major peak eluting at about 43-44% of ethanol contained the title compound. The fractions containing the major peak were pooled, water was added to reduce the ethanol concentration to 20% (v/v), and the pH was adjusted to 5.5. The solution was left overnight at -20° C., whereby the product precipitated. The precipitate was isolated by centrifugation at -8° C. and dried in vacuo. The yield of the title compound was 90 mg.

Molecular mass, found by MS: 5892, theory: 5890.

Example 15

Synthesis of N^{εB29}-(N-myristoyl- α -glutamyl) Human Insulin

500 mg of (A1,B1)-diBoc human insulin was dissolved in 2.5 ml of DMSO and 428 μ l of ethyl diisopropylamine,

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diluted with 2.5 ml of DMSO/DMF 1/1 (v/v), was added. The temperature was adjusted to 15° C. and 85 mg of N-myristoyl-Glu(OBut) N-hydroxysuccinimide ester, dissolved in 2.5 ml of DMSO/DMF 1/1 (v/v), was added. After 30 min the reaction mixture was poured into 60 ml of water, the pH adjusted to 5 and the precipitate isolated by centrifugation. The precipitate was dried in vacuo. The dried reaction mixture was dissolved in 25 ml of TFA, and the solution was left for 30 min at room temperature. The TFA was removed by evaporation in vacuo. The gelatinous residue was dissolved in 60 ml of water and the pH was adjusted to 11.2 using concentrated ammonia. The title compound was crystallized from this solution by adjustment of the pH to 8.51 using 6 N HCl. The product was isolated by centrifugation, washed once by 10 ml of water, and dried in vacuo. Yield 356 mg. Purity by HPLC 94%.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: CH₃(CH₂)₁₂CONHCH(CH₂CH₂COOH)CO—.

Molecular mass, found by MS: 6146, theory: 6148.

Example 16

Synthesis of N^{εB29}-undecanoyl-des(B30) Human Insulin

The title compound was synthesized analogously to N^{εB29}-dodecanoyl des(B30) human insulin as described in Example 14, by using undecanoic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5876, theory: 5876.

Example 17

Synthesis of N^{εB29}-tridecanoyl des(B30) Human Insulin

The title compound was synthesized analogously to N^{εB29}-dodecanoyl des(B30) human insulin as described in Example 14, by using tridecanoic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5899, theory: 5904.

Example 18

Synthesis of N^{εB29}-myristoyl des(B30) Human Insulin

The title compound was synthesized analogously to N^{εB29}-dodecanoyl des(B30) human insulin as described in Example 14, by using myristic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5923, theory: 5918.

Example 19

Synthesis of N^{εB29}-palmitoyl des(B30) Human Insulin

The title compound was synthesized analogously to N^{εB29}-dodecanoyl des(B30) human insulin as described in Example 14, by using palmitic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5944, theory: 5946.

Example 20

Synthesis of N^{εB29}-suberoyl-D-thyroxine Human Insulin

a. Preparation of N-(succinimidylsuberoyl)-D-thyroxine

Disuccinimidyl suberate (1.0 g, Pierce) was dissolved in DMF (50 ml), and D-thyroxine (2.0 g, Aldrich) was added with stirring at 20° C. The thyroxine slowly dissolved, and after 20 hours the solvent was removed by evaporation in vacuo. The oily residue was crystallized from 2-propanol to yield 0.6 g of N-(succinimidylsuberoyl)-D-thyroxine, m.p. 128–133° C.

b. Reaction of (A1,B1)-diBoc Human Insulin with N-(succinimidylsuberoyl)-D-thyroxine

(A1,B1)-diBoc human insulin (200 mg) was dissolved in dry DMF (10 ml) by addition of triethylamine (20 μl) at room temperature. Then, N-(succinimidylsuberoyl)-D-thyroxine (80 mg) was added. The reaction was monitored by reversed phase HPLC and when the reaction was about 90% complete, the solvent was removed in vacuo. To the evaporation residue, anhydrous trifluoroacetic acid (5 ml) was added, and the solution was kept for 1 hour at room temperature. After removal of the trifluoroacetic acid in vacuo, the residue was dissolved in a mixture of 1M acetic acid (5 ml) and acetonitrile (1.5 ml), purified by preparative reversed phase HPLC and desalted on a PD-10 column. The yield of N^{εB29}-suberoyl-D-thyroxine human insulin was 50 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: Thyrox-CO(CH₂)₆CO—, wherein Thyrox is thyroxine which is bound to the octanedioic acid moiety via an amide bond to its α-amino group.

Molecular mass of the product found by MS: 6724, theory: 6723.

Example 21

Synthesis of N^{εB29}-(2-succinylamido)myristic acid Human Insulin

a. Preparation of α-aminomyristic acid methyl ester, HCl

To methanol (5 ml, Merck) at -10° C., thionyl chloride (0.2 ml, Aldrich) was added dropwise while stirring vigorously. Then, α-aminomyristic acid (0.7 g, prepared from the α-bromo acid by reaction with ammonia) was added. The reaction mixture was stirred at room temperature overnight, and then evaporated to dryness. The crude product (0.7 g) was used directly in step b.

b. Preparation of N-succinoyl-α-aminomyristic acid methyl ester

α-Aminomyristic acid methyl ester, HCl (0.7 g) was dissolved in chloroform (25 ml, Merck). Triethylamine (0.35 ml, Fluka) was added, followed by succinic anhydride (0.3 g, Fluka). The reaction mixture was stirred at room temperature for 2 hours, concentrated to dryness, and the residue recrystallized from ethyl acetate/petroleum ether (1/1). Yield: 0.8 g.

c. Preparation of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester

N-succinoyl-α-aminomyristic acid methyl ester (0.8 g) was dissolved in dry DMF (10 ml, Merck, dried over 4 Å molecular sieve). Dry pyridine (80 μl, Merck), and di(N-succinimidyl)carbonate (1.8 g, Fluka) were added, and the reaction mixture was stirred overnight at room temperature. The evaporation residue was purified by flash chromatog-

raphy on silica gel 60 (Merck), and recrystallized from 2-propanol/petroleum ether (1/1). Yield of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester: 0.13 g, m.p. 64–66° C.

d. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester

The reaction was carried out as in Example 20 b., but using N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester (16 mg) instead of N-(succinimidylsuberoyl)-D-thyroxine. After removal of the trifluoroacetic acid in vacuo, the evaporation residue was treated with 0.1M sodium hydroxide at 0° C. to saponify the methyl ester. When the saponification was judged to be complete by reversed phase HPLC, the pH value in the solution was adjusted to 3, and the solution was lyophilized. After purification by preparative reversed phase HPLC and desalting on a PD-10 column, the yield of N^{εB29}-(2-succinylamido)myristic acid human insulin was 39 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: CH₃(CH₂)₁₁CH(COOH)NHC(=O)CH₂CH₂CO—.

Molecular mass of the product found by MS: 6130, theory: 6133.

Example 22

Synthesis of N^{εB29}-octyloxycarbonyl Human Insulin

The synthesis was carried out as in Example 20 b., but using n-octyloxycarbonyl N-hydroxysuccinimide (9 mg, prepared from n-octyl chloroformate (Aldrich) and N-hydroxysuccinimide), instead of N-(succinimidylsuberoyl)-D-thyroxine. The yield of N^{εB29}-octyloxycarbonyl human insulin was 86 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: CH₃(CH₂)₇OCO—.

Molecular mass of the product found by MS: 5960, theory: 5964.

Example 23

Synthesis of N^{εB29}-(2-succinylamido)palmitic acid Human Insulin

a. Preparation of N-(succinimidylsuccinoyl)-α-amino palmitic acid methyl ester

This compound was prepared as described in Example 21 a.-c., using α-amino palmitic acid instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc Human Insulin with N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester

The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester to give N^{εB29}-(2-succinylamido)palmitic acid human insulin.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: CH₃(CH₂)₁₃CH(COOH)NHC(=O)CH₂CH₂CO—.

Example 24

Synthesis of N^{εB29}-(2-succinylamidoethoxy) palmitic acid Human Insulin

a. Preparation of N-(succinimidylsuccinoyl)-2-aminooxy palmitic acid methyl ester

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This compound was prepared as described in Example 21 a.-c. but using 2-aminoethoxy palmitic acid (synthesized by the general procedure described by R. TenBrink, *J. Org. Chem.* 52 (1987) 418-422 instead of α -amino myristic acid. b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-2-aminoethoxypalmitic acid methyl ester

The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-2-aminoethoxypalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester to give N ^{ϵ B29}-(2-succinylamidoethoxy)palmitic acid human insulin.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: CH₃(CH₂)₁₃CH(COOH)NHCH₂CH₂OCOCH₂CH₂CO—.

Example 25

Synthesis of N ^{ϵ B29}-lithocholoyl- α -glutamyl des (B30) Human Insulin

The synthesis was carried out as in Example 13 using N-lithocholoyl-L-glutamic acid α -N-hydroxysuccinimide ester, γ -tert-butyl ester instead of decanoic acid N-hydroxysuccinimide ester.

The product of this example is thus des(B30) human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: lithocholoyl-NHCH(CH₂CH₂COOH)CO—.

Molecular mass of the product found by MS: 6194, theory: 6193.

Example 26

Synthesis of N ^{ϵ B29}-3,3',5,5'-tetraiodothyroacetyl Human Insulin

The synthesis was carried out as in Example 10 using 3,3',5,5'-tetraiodothyroacetic acid N-hydroxysuccinimide ester, instead of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6536, theory: 6538.

Example 27

Synthesis of N ^{ϵ B29}-L-thyroxyl Human Insulin

The synthesis was carried out as in Example 10 using Boc-L-thyroxine N-hydroxysuccinimide ester, instead of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6572, theory: 6567.

Example 28

A Pharmaceutical Composition Comprising 600 nmol/ml of N ^{ϵ B29}-decanoyl des(B30) Human Insulin, 1/3Zn²⁺ in Solution

N ^{ϵ B29}-decanoyl des(B30) human insulin (1.2 μ mol) was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. 0.01 M zinc acetate (60 μ l) and a solution containing 0.75% of phenol and 4% of glycerol (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

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Example 29

A Pharmaceutical Composition Comprising 600 nmol/ml of N ^{ϵ B29}-decanoyl Human Insulin, 1/3Zn²⁺ in Solution

1.2 μ mol of the title compound was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. A solution containing 0.75% of phenol and 1.75% of sodium chloride (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

Example 30

A Pharmaceutical Composition Comprising 600 nmol/ml of N ^{ϵ B29}-lithocholoyl Human Insulin in Solution

1.2 μ mol of the title compound was suspended in water (0.8 ml) and dissolved by adjusting the pH value of the solution to 8.5 using 0.2 M sodium hydroxide. To the solution was then added 0.8 ml of a stock solution containing 0.75% cresol and 4% glycerol in water. Finally, the pH value was again adjusted to 8.5 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

Example 31

A Pharmaceutical Composition Comprising a Solution of 600 nmol/ml of N ^{ϵ B29}-hexadecanoyl Human Insulin 1/3 Zinc Ion per Insulin Monomer, 16 mM m-cresol, 16 mM phenol, 1.6% glycerol, 10 mM sodium chloride and 7 mM sodium phosphate

1.2 μ mol of N ^{ϵ B29}-hexadecanoyl human insulin was dissolved in water (0.5 ml) by addition of 0.2 M sodium hydroxide to pH 8.0 and 40 μ l of 0.01 M zinc acetate was added. To the solution was further added 100 μ l of 0.32 M phenol, 200 μ l of 0.16 M m-cresol, 800 μ l of 4% glycerol, 33.3 μ l of 0.6 M sodium chloride, and 140 μ l of 0.1 M sodium phosphate (pH 7.5). The pH value of the solution was adjusted to 7.5 with 0.1 M hydrochloric acid and the volume adjusted to 2 ml with water.

Example 32

Solubility of Various Compositions Comprising N ^{ϵ B29}-tetradecanoyl des(B30) Human Insulin and N ^{ϵ B29}-hexadecanoyl Human Insulin

The solubility of N ^{ϵ B29}-tetradecanoyl des(B30) human insulin and N ^{ϵ B29}-hexadecanoyl human insulin in different compositions was tested. The compositions were prepared as described in Example 31 with the necessary adjustment of the amount of the components. Zinc acetate was either left out or an amount corresponding to 1/3 Zn²⁺ per insulin monomer was used. Sodium chloride was used in amounts which resulted in a final concentration of 5, 25, 50, 75, 100 or 150 mM of sodium chloride. Zinc-free insulin was added to give a final amount in the composition of 1000 nmol/ml. In some cases a precipitate formed. The resulting solutions and suspensions were kept at 4° C. for a week and the concentration of insulin in solution in each composition was

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then measured by high performance size exclusion chromatography relative to a standard of human insulin (column: Waters ProteinPak 250x8 mm; eluent: 2.5 M acetic acid, 4 mM arginine; 20% acetonitrile; flow rate: 1 ml/min; injection volume: 40 μ l; detection: UV absorbance at 276 nm). The results, in nmol/ml, are given in the table below:

Solubility of insulins (nmol/ml) in 16 mM phenol, 16 mM m-cresol, 1.6% glycerol, 7 mM sodium phosphate, and pH 7.5, varying zinc acetate and

sodium chloride (mM) concentrations at 4° C.	Sodium chloride					
	5 mM	25 mM	50 mM	75 mM	100 mM	150 mM
N ^ε B ²⁹ -tetradecanoyl des(B30) human insulin, zinc-free.	82	115	54	77	74	84
N ^ε B ²⁹ -tetradecanoyl des(B30) human insulin, 1/3 Zn ²⁺ per insulin monomer.	>950	>950	>950	>950	>950	485
N ^ε B ²⁹ -hexadecanoyl human insulin, zinc-free.	>890	>950	283	106	45	29
N ^ε B ²⁹ -hexadecanoyl human insulin, 1/3 Zn ²⁺ per insulin monomer.	>950	>950	>950	>950	920	620

In conclusion it appears that the solubility of the acylated insulins is increased by the addition of zinc. This is contrary to published data on human, porcine and bovine insulin (J Brange: *Galenics of Insulin*, page 19, Springer Verlag (1987); J Markussen et al. *Protein Engineering* 1 (1987) 205-213).

Example 33

Preparative Crystallization of Zinc-Free N^εB²⁹-tetradecanoyl des(B30) Human Insulin

10 g of N^εB²⁹-tetradecanoyl des(B30) human insulin was dissolved in 120 ml of 0.02 M NH₄Cl buffer adjusted to pH 9.0 with NH₃ in ethanol/water (1:4, v/v). Gentle stirring was maintained throughout the crystallization. Crystallization was initiated at 23° C. by addition of 20 ml of 2.5 M NaCl dissolved in ethanol/water (1:4, v/v). A slight turbidity appeared in the solution. Further, 20 ml of 2.5 M sodium chloride dissolved in ethanol/water (1:4, v/v) was added at a constant rate of 5 ml/h, which caused the crystallization to proceed slowly. In order to decrease the solubility of the insulin, the pH value was then adjusted to 7.5 using 1 N hydrochloric acid. Finally, the temperature was lowered to 4° C. and the stirring continued overnight. The crystals were collected by filtration, washed twice with 25 ml of 0.2 M NaCl in ethanol/water (1:4, v/v), sucked dry and lyophilized.

The weight of the wet filter cake was 19.33 g.

The weight of lyophilized filter cake was 9.71 g.

Example 34

Synthesis of Lys^{B29}(N^ε-[N^α-tetradecanoyl-Glu-Gly-1] des(B30) Human Insulin

500 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 186 μ l of 4-methylmorpholine and 3814 μ l of DMSO. The reaction was initiated by addition of 144 mg of tetradecanoyl-Glu(γ -OtBu)-Gly-OSu dissolved in 1000 μ l of DMF. The reaction conducted at 15° C. and it was stopped after 4.5 hours by addition of 100 ml of acetone. The reaction product precipitated by addition of a few drops of concentrated HCl was subsequently isolated by centrifuga-

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tion. The precipitate was then suspended in 100 ml of acetone, isolated by centrifugation and dried in vacuum. 637 mg of material was obtained.

The Boc protecting groups were eliminated by addition of 5 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 100 ml of acetone and a few drops of concentrated HCl. The precipitate was then suspended in 100 ml acetone and isolated by centrifugation. The precipitated material was dissolved in 200 ml of 25% ethanol at pH 8 by addition of NH₄OH and purified by reversed phase HPLC. The dissolved material was applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μ m, pore size 100 Å) and equilibrated with 0.02 M Bis-Tris, 30% ethanol adjusted to pH 7.3 with hydrochloric acid at a temperature of 40° C. The elution was performed using mixtures of 70% ethanol in water and Bis-Tris buffer. The flow was 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 50% and the effluent was monitored by its UV absorbance at 280 nm. The appropriate fraction was diluted to 20% ethanol adjusted to pH 4.5 and frozen at -20° C. The precipitated material was isolated after equilibration of the sample at 1° C. and subsequent centrifugation at the same temperature. The precipitate was dried in vacuum. Thus 292 mg of the title compound was obtained at a purity of 95.5%.

Molecular mass, found by MS: 6102 \pm 6, theory: 6103.

The lipophilicity of the title compound, relative to human insulin, k'_{rel} =20. The determination was carried out as described on page 23 of the description.

The disappearance half-life, T_{50%}, of the title compound after subcutaneous injection in pigs was found to be 11.9 hours. The determination was carried out as described on page 24 of the description using a composition similar to those described in Table 2 on page 26 of the description.

Example 35

Synthesis of Lys^{B29}(N^ε-tetradecanoyl-Glu-) des(B30) Human Insulin

500 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 186 μ l of 4-methylmorpholine and 3814 μ l of DMSO. The reaction was initiated by addition of 85 mg of N^α-tetradecanoyl-Glu(OtBu)-OSu dissolved in 1000 μ l of DMF. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The intermediate product was isolated and the protection groups were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 356 mg of the title compound was obtained at a purity of 94.1%. Molecular mass, found by MS: 6053 \pm 6, theory: 6046.

The lipophilicity of the title compound, relative to human insulin, k'_{rel} =24. The determination was carried out as described on page 23 of the description.

The disappearance half-life, T_{50%}, of the title compound after subcutaneous injection in pigs was found to be 8.8 hours. The determination was carried out as described on page 24 of the description using a composition similar to those described in Table 2 on page 26 of the description.

Example 36

Synthesis of Lys^{B29}(N^ε-[N^α-tetradecanoyl-Glu(-)-OH]) Human Insulin

400 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 232 μ l of ethyldiisopropylamine, 1880 μ l of

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DMSO and 2088 μ l of 1-methyl-2-pyrrolidone. The reaction was initiated by addition of 138 mg of N $^{\alpha}$ -tetradecanoyl-Glu(OSu)-OtBu dissolved in 800 μ l of 1-methyl-2-pyrrolidone. The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups were removed from the intermediate product by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 222 mg of the title compound was obtained at a purity of 95.5%. Molecular mass, found by MS: 6150 \pm 6, theory: 6147.

The lipophilicity of the title compound, relative to human insulin, k'_{ret} =21. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 8.0 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 37

Synthesis of Lys B29 (N $^{\epsilon}$ -[N $^{\alpha}$ -hexadecanoyl-Glu(-)-OH]) Human Insulin

400 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 232 μ l of ethyldiisopropylamine, 880 μ l of DMSO and 2088 μ l of 1-methyl-2-pyrrolidone. The reaction was initiated by addition of 73 mg of N $^{\alpha}$ -hexadecanoyl-Glu(OSu)-OtBu dissolved in 800 μ l of DMF. The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 476 mg of intermediate product was obtained. The protection groups were removed from the intermediate product by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 222 mg of the title compound was obtained at a purity of 81.2%. Molecular mass, found by MS: 6179 \pm 6, theory: 6175.

The lipophilicity of the title compound, relative to human insulin, k'_{ret} =67. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 13.0 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 38

Synthesis of Lys B29 (N $^{\epsilon}$ -[N $^{\alpha}$ -octadecanoyl-Glu(-)-OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 232 μ l of ethyldiisopropylamine, 3000 μ l of DMSO and 268 μ l of dimethylformamide. The reaction was initiated by addition of 114 mg N $^{\alpha}$ -octadecanoyl-Glu(OSu)-OtBu dissolved in 500 μ l of DMF. The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 420 mg of intermediate product was obtained. The protection groups were removed from the intermediate product by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 169 mg of the title compound was obtained at a purity of 98.3%. Molecular mass, found by MS: 6103 \pm 5, theory: 6102.

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The lipophilicity of the title compound, relative to human insulin, k'_{ret} =185. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 9.7 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 39

Synthesis of Lys B29 (N $^{\epsilon}$ -[N $^{\alpha}$ -tetradecanoyl-Glu(-)-OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 232 μ l of ethyldiisopropylamine and 3000 μ l of DMSO. The reaction was initiated by addition of 138 mg of N $^{\alpha}$ -tetradecanoyl-Glu(OSu)-OtBu dissolved in 768 μ l of DMF. The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 505 mg of intermediate product was obtained. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 237 mg of the title compound was obtained at a purity of 96.7%. Molecular mass, found by MS: 6053 \pm 6, theory: 6046.

The lipophilicity of the title compound, relative to human insulin, k'_{ret} =21. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 12.8 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 40

Synthesis of Lys B29 (N $^{\epsilon}$ -[N $^{\alpha}$ -hexadecanoyl-Glu(-)-OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 232 μ l of ethyldiisopropylamine, 3000 μ l of DMSO and 400 μ l of dimethylformamide. The reaction was initiated by addition of 73 mg of N $^{\alpha}$ -hexadecanoyl-Glu(OSu)-OtBu dissolved in 400 μ l of DMF. The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 153 mg of the title compound was obtained at a purity of 95.2%. Molecular Mass, found by MS: 6073 \pm 6, theory: 6074.

The lipophilicity of the title compound, relative to human insulin, k'_{ret} =67. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 18.0 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 41

Synthesis of Lys B29 (N $^{\epsilon}$ -[N $^{\alpha}$ -lithocholyl-Glu(-)-OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 148 μ l 4-methylmorpholine and

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3452 μ l of DMSO. The reaction was initiated by addition of 132 mg of N $^{\alpha}$ -lithocholoyl-Glu(OSu)-OtBu dissolved in 400 μ l of DMF. The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 493 mg of intermediate product was obtained. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 209 mg of the title compound was obtained at a purity of 97.4%. Molecular Mass, found by MS: 6185 \pm 10, theory: 6194.

Example 42

Lys^{H29}(N $^{\epsilon}$ -N $^{\alpha}$ -tetradecanoyl Aad(-)-OH) des
(B30) Human Insulin

Aad is 5-aminohexadioic acid. 347 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 129 μ l of 4-methylmorpholine and 2645 μ l of DMSO. The reaction was initiated by addition of 58 mg of N $^{\alpha}$ -tetradecanoyl-Aad(OSu)-OtBu dissolved in 694 μ l of DMF. The activated ester was prepared in analogy with chemistry well-known from aspartic acid derivatisation (L. Benoiton: Can.J.Chem.40,570-72,1962, R. Roeske: J.Org.Chem 28 1251-93 (1963)). The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

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Thus 149 mg of the title compound was obtained at a purity of 97.9%. Molecular Mass, found by MS: 6061 \pm 2, theory: 6060.

The lipophilicity of the title compound, relative to human insulin, k'_{rel} =21. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 16.1 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 43

Synthesis of Lys^{H29}(N $^{\epsilon}$ -[N $^{\alpha}$ -tetradecanoyl- γ -carboxy-Glu-]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 190 μ l of triethylamine and 3000 μ l of DMSO. The reaction was initiated by addition of 83 mg of γ -carboxy Glu N-tetradecansyre γ,γ' -di(OtBu) α -(OSu) (i.e. (tBuOCO)₂CHCH₂-CH(COOSu)-NH-CO(CH₂)₁₂CH₃) dissolved in 800 μ l of DMF. The reaction was conducted at 15 $^{\circ}$ C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

63 mg of the title compound were obtained. Molecular Mass, found by MS: 6090 \pm 3, theory: 6091.

The lipophilicity of the title compound, relative to human insulin, k'_{rel} =10. The determination was carried out as described on page 23 of the description.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 49

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1 5 10 15

Glu Asn Tyr Cys Xaa
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Val Xaa Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr

-continued

1	5	10	15
Leu	Val	Cys	Gly
	Glu	Arg	Gly
		Phe	Phe
		Tyr	Thr
		Pro	Lys
		Xaa	
	20	25	30

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 110 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGCTAAGAG ATTCGTTGAC CAACACTTGT GCGGTTCTCA CTGGTTGAA GCTTGTACT 60
 TGGTTTGTTG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 100 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGCGGGCTG CGTCTAAGCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60
 AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCGCCATGG CTAAGAGATT CGTTG 25

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCTCTAGA GCCTGCGGGC TGCCTCT 27

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 110 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGCTAAGAG ATTCGTTACT CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60
 TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCGCCATGG CTAAGAGATT CGTTA 25

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 100 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCGGGCTG CGTCTAACCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60
 AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACGTACGTTT TAGAGCCTGC GGGCTGC 27

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 78 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTTGGTTG AAGCTTTGTA CTTGGTTTGT GGTGAAAGAG GTTCTTCTA CACTCCAAAG 60
 ACTAGAGGTA TCGTTGAA 78

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 63 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCTG AAGCTAGATT CGTTAACCAA 60

CAC 63

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 65 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCGA AGCTGAAAGA TTCGTTAACC 60

AACAC 65

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 415 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AACTATCAA TTTCATACAC 60

AATATAAAGC ACCAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC 112

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
1 5 10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG 160

Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
15 20 25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC 208

Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
30 35 40

GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC 256

Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His
45 50 55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC 304

Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60 65 70 75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT 352

Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
80 85 90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT AAC TAGACGAGC 401

Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
95 100

CCGCAGGCTC TAGA 415

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 104 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1           5           10           15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20           25           30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35           40           45
Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50           55           60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65           70           75           80
Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85           90           95
Tyr Gln Leu Glu Asn Tyr Cys Asn
100

```

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 415 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

TAGCTTAAAG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTGATAGTT AAAGTATGTG      60
TTATATTTCG TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC      120
GACCCGGGTT GGTCACTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA      180
GTAGCGGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAGAA      300
GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC      360
AAGAAACATG GTTAACCTTT TGATGACATT GATCTGCGTC GGGCGTCCGA GATCT      415

```

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 523 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```

ATCGAATCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAAA TTTCATACAC      60
AATATAAAGC ATTAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      112
          Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
          1           5           10
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Glu
          15           20           25

```

-continued

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp 30 35 40	208
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr 45 50 55	256
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala 60 65 70 75	304
AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA GAA GTT AAC CAA CAC TTG Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu 80 85 90	352
TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg 95 100 105	400
GGT TTC TTC TAC ACT GAA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA Gly Phe Phe Tyr Thr Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu 110 115 120	448
CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys 125 130 135	496
AAC TAGACGCAGC CCGCAGGCTC TAGA Asn 140	523

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser 1 5 10 15
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln 20 25 30
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe 35 40 45
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu 50 55 60
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val 65 70 75 80
Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu Cys Gly Ser His Leu 85 90 95
Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr 100 105 110
Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser 115 120 125
Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn 130 135 140

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG    60
TTATATTTCG TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG    120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG    180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG    240
TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTGTAACG    300
ACGATTTCTT CTTCGCCATA GAAACCTATT CTCCTTCAA TTGGTTGTGA ACACGCCAAG    360
AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGACTTTT    420
CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT    480
TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT                        523

```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

ATCGAATTC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAAA TTTCATACAC    60
AATATAAAGC ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC    112
      Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
      1           5           10
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG    160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
      15           20           25
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC    208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
      30           35           40
GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC    256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
      45           50           55
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC    304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
      60           65           70           75
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT    352
Thr Pro Lys Ser Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
      80           85           90
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC    401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
      95           100
CCGCAGGCTC TAGA                        415

```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1 5 10 15
 Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20 25 30
 Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45
 Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60
 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80
 Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95
 Tyr Gln Leu Glu Asn Tyr Cys Ala
 100

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG 60
 TTATATTGCG TGGTITTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC 120
 GACCCGGGTT GGTCACTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA 180
 GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT 240
 GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCTAAGAA 300
 GATGTGAGGT TTCAGACTGC TCGGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC 360
 AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT 415

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAATATCAA TTTCATACAC 60
 AATATAACG ACCAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC 112
 Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
 1 5 10
 GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG 160
 Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
 15 20 25
 ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC 208
 Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn

-continued

30	35	40	
GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC			256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His			
45	50	55	
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC			304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr			
60	65	70	75
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT			352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr			
80	85	90	
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC			401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala			
95	100		
CGGCAGGCTC TAGA			415

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1 5 10 15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20 25 30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95

Tyr Gln Leu Glu Asn Tyr Cys Ala
 100

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTGATAGTT AAAGTATGTG 60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAG AACAGGAAGT AGCCTAAGAC 120

GACCCGGGTT GGTCACTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA 180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT 240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAGAA 300

GATGTAGGT TTCAGACTGC TCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC 360

AAGAAACATG GTTAACCTTT TGATGACACG ANTCTGCGTC GGGCGTCCGA GATCT 415

-continued

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```

ATCGAATTC ATTCAAGAAAT AGTTCAAACA AGAAGATTAC AAATATCAA TTTCATACAC      60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
      Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
      1              5              10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
      15              20              25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
      30              35              40

GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
      45              50              55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
      60              65              70              75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
      80              85              90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCGAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
      95              100

CCGCAGGCTC TAGA      415

```

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 104 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1              5              10              15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20              25              30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35              40              45
Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50              55              60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65              70              75              80
Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85              90              95
Tyr Gln Leu Glu Asn Tyr Cys Gly

```

-continued

100

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTCG TGGTTTTCCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC      120
GACCCGGGTT GGTCACTGAC CGTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA      180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCAAAGAA      300
GATGTGAGGT TTCAGACTGC TGCAGATCCC ATAGCAACTT GTTACAACAT GAAGATAGAC      360
AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT      415

```

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC      60
AATATAAAGC ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
      Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
          1             5             10
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
          15             20             25
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
          30             35             40
GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
          45             50             55
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
          60             65             70             75
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
          80             85             90
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
          95             100
CCGCAGGCTC TAGA      415

```

(2) INFORMATION FOR SEQ ID NO:30:

-continued

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 104 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1             5             10             15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
      20             25             30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
      35             40             45
Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
      50             55             60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
      65             70             75             80
Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
      85             90             95
Tyr Gln Leu Glu Asn Tyr Cys Gly
      100

```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```

TAGCTTAAGG TAAGTTCCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAAGT AGCCTAAGAC      120
GACCCGGGTT GGTCACTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA      180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA      300
GATGTGAGGT TTCAGACTGC TCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC      360
AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT      415

```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 523 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```

ATCGAATTC ATTCAAGAAT AGTTCAACA AGAAGATTAC AAATATCAA TTTCATACAC      60
AATATAAAGC ATTAAGAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      112
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
      1             5             10

```


6,011,007

61

62

-continued

```

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA 160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
      15                20                25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT 208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
      30                35                40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA 256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
      45                50                55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT 304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
      60                65                70                75

AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA TTC GTT AAC CAA CAC TTG 352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu
      80                85                90

TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA 400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
      95                100                105

GGT TTC TTC TAC ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA 448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
      110                115                120

CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT 496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
      125                130                135

AAC TAGACGCAGC CCGCAGGCTC TAGA 523
Asn
140

```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1           5           10           15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
      20           25           30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
      35           40           45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
      50           55           60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
      65           70           75           80

Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
      85           90           95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
      100          105          110

Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
      115          120          125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
      130          135          140

```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 523 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```

TAGCTTAAAG TAAGTTCCTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTCG TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAA ATAAGCGTCG      120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG      180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG      240
TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG      300
ACGATTTCTT CTCCCCATA GAAACCTATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG      360
AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAGAAGA TGTGAGGTTT      420
CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT      480
TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT                          523
  
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 409 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 80..385

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```

ATCGAATTC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC      60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
          Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
          1             5             10
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
          15             20             25
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
          30             35             40
GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His
          45             50             55
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
          60             65             70             75
ACT CCT AAG GAA AAG AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC      352
Thr Pro Lys Glu Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile
          80             85             90
TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC CCGCAGGCTC      405
Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
          95             100
TAGA                                                                409
  
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

-continued

- (A) LENGTH: 102 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1           5           10          15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
          20          25          30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
          35          40          45
Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
          50          55          60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Glu Lys
          65          70          75          80
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
          85          90          95
Leu Glu Asn Tyr Cys Gly
          100

```

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 409 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TGGTTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAAGT AGCCTAAGAC      120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACCTC TAAGGCCTTC TCAGAGACTA      180
GTAGCGCACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAGAA      300
GATGTGAGGA TTCTTTTCTT CTCCATAGCA ACTTGTTACA ACATGAAGAT AGACAAGAAA      360
CATGTTTAAC CTTTGTGATG CACCAATCTG CGTCGGGCGT CCGAGATCT      409

```

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 511 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 77..487

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```

GAATTCATT CAAGAATAGT TCAACAAGA AGATTACAAA CTATCAATTT CATAACAAT      60
ATAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      109
          Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
          1           5           10
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      157

```


-continued

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```

CTTAAGGTAA GTTCTTATCA AGTTTGTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA    60
TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAATAATGA CGTCAAAATA AGCGTCGTAG    120
GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG    180
ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCTAAAG CTACAACGAC AAAACGGTAA    240
AAGGTTGTCG TGTATTATGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG    300
ATTCTTCTCT CCCCATAGGT ACCGATTCTC TAAGCAATIG GTTGTGAACA CGCCAAGGGT    360
GAACCAACTT CGAAACATGA ACCAAACACC ACTTTCTCCA AAGAAGATGT GAGGTTTCTG    420
ATCTCCATAG CAACTTGTTA CAACATGAAG ATAGACAAGA AACATGGTTA ACCTTTTGAT    480
GACGTTGATC TGCCTCGGGC GTCGAGATC T                                     511

```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 523 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```

ATCGAATCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATATCAA TTTCATACAC    60
AATATAAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA    112
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
              1             5             10
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA    160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
              15             20             25
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT    208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
              30             35             40
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA    256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
              45             50             55
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT    304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
              60             65             70             75
AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG    352
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
              80             85             90
TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGC GGT GAA AGA    400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
              95             100             105
GGT TTC TTC TAC ACT CCT AAG TCT GAC GAT GCT AAG GGT ATT GTC GAG    448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
              110             115             120
CAA TGC TGT ACC TCC ATC TGC TCC TTG TAC CAA TTG GAA AAC TAC TGC    496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
              125             130             135
AAC TAGACGCAGC CCGCAGGCTC TAGA                                     523
Asn

```

-continued

140

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1           5           10           15
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
          20           25           30
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
          35           40           45
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
          50           55           60
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
          65           70           75           80
Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
          85           90           95
Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
          100          105          110
Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
          115          120          125
Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
          130          135          140

```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```

TAGCTTAAGG TAAGTCTTAA TCAAGTTTGT TCTTCTAATG TTGATAGTT AAAGTATGTG      60
TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG      120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG      180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG      240
TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTIA TGATGATAAC GGTCGTAACG      300
ACGATTTCCT CTCCCCATA GGTACCGATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG      360
GGTGAACCAA CTTCGAAACA TGAACCAAAC GCCACTTTCT CCAAAGAAGA TGTGAGGATT      420
CAGACTGCTA CGATTCCCAT AACAGCTCGT TACGACATGG AGGTAGACGA GGAACATGGT      480
TAACCTTTTG ATGACGTTGA TCTGCGTCGG GCGTCCGAGA TCT                        523

```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 535 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 77..511

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT      60
ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      109
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
      1             5             10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
      15             20             25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
      30             35             40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
      45             50             55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
      60             65             70             75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA      349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu
      80             85             90

GCT AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA GCT      397
Ala Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala
      95             100             105

TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG ACT      445
Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr
      110             115             120

AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA      493
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
      125             130             135

TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA      535
Leu Glu Asn Tyr Cys Asn
      140             145

```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 145 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1             5             10             15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
      20             25             30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
      35             40             45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
      50             55             60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
      65             70             75             80

Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Arg Phe Val Asn
      85             90             95

```

-continued

Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
 100 105 110
 Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu
 115 120 125
 Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
 130 135 140
 Asn
 145

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 535 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CTTAAGGTAA GTTCTTATCA AGTTTGTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA 60
 TATTGCTAA TTTTCTTACT CTAAAGGAAG TTAATAATGA CGTCAAATA AGCGTCGTAG 120
 GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGCTTCTA CTTTGCCGTG TTTAAGGCCG 180
 ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAG CTACAACGAC AAAACGGTAA 240
 AAGGTTGTCG TGTATTATGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG 300
 ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTGACTTC GATCTAAGCA 360
 ATTGGTTGTG AACACGCCAA GGGTGAACCA ACTTCGAAAC ATGAACCAA CACCACTTTC 420
 TCCAAAGAAG ATGTGAGGTT TCTGATCTCC ATAGCAACTT GTTACAACAT GAAGATAGAC 480
 AAGAAACATG GTTAACCTTT TGATGACGTT GATCTGCGTC GGGCGTCCGA GATCT 535

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 538 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 77..514

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT 60
 ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA 109
 Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
 1 5 10
 TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA 157
 Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
 15 20 25
 GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT 205
 Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
 30 35 40
 TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA 253
 Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
 45 50 55
 AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT 301

-continued

Asn	Asn	Gly	Leu	Leu	Phe	Ile	Asn	Thr	Thr	Ile	Ala	Ser	Ile	Ala	Ala		
60					65				70					75			
AAA	GAA	GAA	GGG	GTA	TCC	ATG	GCT	AAG	AGA	GAA	GAA	GCT	GAA	GCT	GAA	349	
Lys	Glu	Glu	Gly	Val	Ser	Met	Ala	Lys	Arg	Glu	Glu	Ala	Glu	Ala	Glu		
			80						85					90			
GCT	GAA	AGA	TTC	GTT	AAC	CAA	CAC	TTG	TGC	GGT	TCC	CAC	TTG	GTT	GAA	397	
Ala	Glu	Arg	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu		
			95					100					105				
GCT	TTG	TAC	TTG	GTT	TGT	GGT	GAA	AGA	GGT	TTC	TTC	TAC	ACT	CCA	AAG	445	
Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys		
	110						115					120					
ACT	AGA	GGT	ATC	GTT	GAA	CAA	TGT	TGT	ACT	TCT	ATC	TGT	TCT	TTG	TAC	493	
Thr	Arg	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr		
	125					130					135						
CAA	TTG	GAA	AAC	TAC	TGC	AAC	TAGACGCAGC	CCGCAGGCTC	TAGA							538	
Gln	Leu	Glu	Asn	Tyr	Cys	Asn											
140					145												

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 146 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Met	Arg	Phe	Pro	Ser	Ile	Phe	Thr	Ala	Val	Leu	Phe	Ala	Ala	Ser	Ser		
1				5				10						15			
Ala	Leu	Ala	Ala	Pro	Val	Asn	Thr	Thr	Thr	Glu	Asp	Glu	Thr	Ala	Gln		
			20					25					30				
Ile	Pro	Ala	Glu	Ala	Val	Ile	Gly	Tyr	Ser	Asp	Leu	Glu	Gly	Asp	Phe		
		35					40					45					
Asp	Val	Ala	Val	Leu	Pro	Phe	Ser	Asn	Ser	Thr	Asn	Asn	Gly	Leu	Leu		
	50					55					60						
Phe	Ile	Asn	Thr	Thr	Ile	Ala	Ser	Ile	Ala	Ala	Lys	Glu	Glu	Gly	Val		
	65				70				75					80			
Ser	Met	Ala	Lys	Arg	Glu	Glu	Ala	Glu	Ala	Glu	Ala	Glu	Arg	Phe	Val		
			85					90					95				
Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val		
		100					105					110					
Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr	Arg	Gly	Ile	Val		
	115						120					125					
Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr		
	130					135					140						
Cys	Asn																
145																	

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 538 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

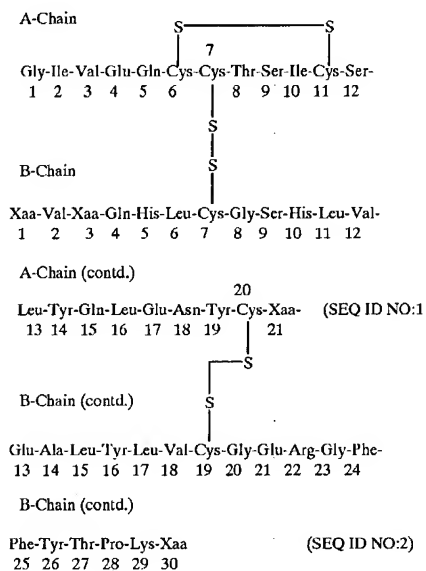
CTTAAGGTAA	GTTCTTATCA	AGTTTGTCT	TCTAATGTTT	GATAGTTAA	GTATGTGTTA	60
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-continued

TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAATAATGA CGTCAAAATA AGCGTCGTAG	120
GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG	180
ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA	240
AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG	300
ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTGCACTTC GACTTTCTAA	360
GCAATTGGTT GTGAACACGC CAAGGGTGAA CCAACTTCGA AACATGAACC AAACACCACT	420
TTCTCCAAAG AAGATGTGAG GTTCTGTATC TCCATAGCAA CTTGTTACAA CATGAAGATA	480
GACAAGAAAC ATGGTTAACC TTTTGATGAC GTTGATCTGC GTCGGGCGTC CGAGATCT	538

We claim:

1. An insulin derivative having the following sequence:



wherein

- Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;
- Xaa at position B1 is Phe or is deleted;
- Xaa at position B30 is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; and
- the ϵ -amino group of Lys^{B29} is substituted with a lipophilic substituent having at least 10 carbon atoms; wherein the insulin derivative is a Zn²⁺ complex and the Zn²⁺ complex of the insulin derivative is more water soluble than the insulin derivative without Zn²⁺.

2. The insulin derivative according to claim 1, wherein Xaa at position A21 is Asn.

3. The insulin derivative according to claim 2, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

4. The insulin derivative according to claim 1, wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser.

5. The insulin derivative according to claim 4, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

6. The insulin derivative according to claim 1, wherein Xaa at position B1 is deleted.

7. The insulin derivative according to claim 6, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

8. The insulin derivative according to claim 1, wherein Xaa at position B1 is Phe.

9. The insulin derivative according to claim 8, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

10. The insulin derivative according to claim 1, wherein Xaa at position B3 is Asn, Asp, Gln or Thr.

11. The insulin derivative according to claim 10, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

12. The insulin derivative according to claim 1, wherein Xaa at position B30 is Ala or Thr.

13. The insulin derivative according to claim 12, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

14. The insulin derivative according to claim 1, wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser, Xaa at position B3 is Asn, Asp, Gln or Thr, and Xaa at position B30 is Ala or Thr.

15. The insulin derivative according to claim 14, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

16. The insulin derivative according to claim 1, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, Xaa at position B1 is Phe and Xaa at position B30 is Thr.

17. The insulin derivative according to claim 16, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

18. The insulin derivative according to claim 1, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

19. The insulin derivative according to claim 1 which is in the form of a hexamer.

20. The insulin derivative according to claim 19, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

21. The insulin derivative according to claim 19, wherein Xaa at position A21 is Asn, Xaa at position B1 is Phe, Xaa at position B3 is Asn, and Xaa at position B30 is Thr.

22. The insulin derivative according to claim 19, wherein two zinc ions bind to the hexamer.

23. The insulin derivative according to claim 22, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

24. The insulin derivative according to claim 19, wherein three zinc ions bind to the hexamer.

25. The insulin derivative according to claim 24, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

26. The insulin derivative according to claim 19, wherein four zinc ions bind to the hexamer.

27. The insulin derivative according to claim 26, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

28. A pharmaceutical composition which is an aqueous solution, comprising (a) an insulin derivative according to claim 1, (b) an isotonic agent, (c) a preservative and (d) a buffer.

29. The pharmaceutical composition according to claim 28, wherein the pH of the aqueous solution is in the range of 6.5-8.5.

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30. The pharmaceutical composition according to claim 28, wherein the solubility of the insulin derivative exceeds 600 nmol/ml of the aqueous solution.

31. The pharmaceutical composition according to claim 28, further comprising an insulin or an insulin analogue which has a rapid onset of action.

32. The pharmaceutical composition according to claim 28, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, Xaa at position B1 is Phe and Xaa at position B30 is Thr.

33. The pharmaceutical composition according to claim 28, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

34. The pharmaceutical composition according to claim 28, wherein the insulin derivative is in the form of a hexamer.

35. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition according to claim 28.

36. The insulin derivative of claim 1, wherein the lipophilic substituent is cyclohexylvaleroyl.

37. The insulin derivative of claim 1, wherein the lipophilic substituent is acyl-glutamyl wherein the acyl is a linear, saturated acyl having 6 to 24 carbon atoms.

38. The insulin derivative of claim 1, wherein the lipophilic substituent is lauroyl.

39. The insulin derivative of claim 1, wherein the lipophilic substituent is myristoyl.

40. The insulin derivative of claim 1, wherein the lipophilic substituent is palmitoyl.

41. The insulin derivative of claim 1, wherein the lipophilic substituent is 2-succinylamido myristic acid.

42. The insulin derivative of claim 1, wherein the lipophilic substituent is 2-succinylamido palmitic acid.

43. The insulin derivative of claim 1, wherein the lipophilic substituent is 2-succinylamidoethyloxy palmitic acid.

44. The insulin derivative of claim 1, wherein the lipophilic substituent is myristoyl- α -glutamyl.

45. The insulin derivative of claim 1, wherein the lipophilic substituent is myristoyl- α -glutamyl-glycyl.

46. The insulin derivative of claim 1, wherein the lipophilic substituent is choloyl.

47. The insulin derivative of claim 1, wherein the lipophilic substituent is 7-deoxycholoyl.

48. The insulin derivative of claim 1, wherein the lipophilic substituent is lithocholoyl.

49. The insulin derivative of claim 1, wherein the lipophilic substituent is lithocholoyl-glutamyl.

50. The insulin derivative of claim 1, wherein the lipophilic substituent is 4-benzoyl-phenylalanine.

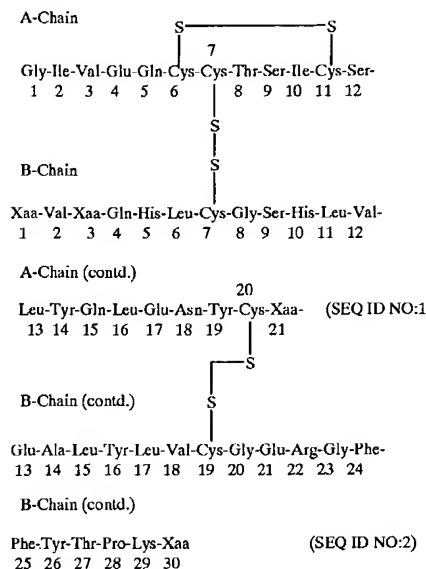
51. The insulin derivative of claim 1, wherein the lipophilic substituent is L-thyroxy.

52. The insulin derivative of claim 1, wherein the lipophilic substituent is suberoyl-D-thyroxine.

53. The insulin derivative of claim 1, wherein the lipophilic substituent is 3,3',5,5'-tetraiodothyroacetyl.

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54. An insulin derivative having the following sequence:



wherein

- (a) Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;
- (b) Xaa at position B1 is Phe or is deleted;
- (c) Xaa at position B30 is deleted; and
- (d) the ϵ -amino group of Lys²⁹ is substituted with a lipophilic substituent having at least 10 carbon atoms; wherein the insulin derivative is a Zn^{2+} complex and the Zn^{2+} complex of the insulin derivative is more water soluble than the insulin derivative without Zn^{2+} .

55. The insulin derivative according to claim 54, wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser.

56. The insulin derivative according to claim 55, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

57. The insulin derivative according to claim 54, wherein Xaa at position B1 is deleted.

58. The insulin derivative according to claim 57, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

59. The insulin derivative according to claim 54, wherein Xaa at position B1 is Phe.

60. The insulin derivative according to claim 59, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

61. The insulin derivative according to claim 54, wherein Xaa at position B3 is Asn, Asp, Gln or Thr.

62. The insulin derivative according to claim 61, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

63. The insulin derivative according to claim 54 wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser, and Xaa at position B3 is Asn, Asp, Gln or Thr.

64. The insulin derivative according to claim 63, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

65. The insulin derivative according to claim 54, wherein Xaa at position A21 is Asn, Xaa at position B1 is Phe, and Xaa at position B3 is Asn.

66. The insulin derivative according to claim 65, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

67. The insulin derivative according to claim 54, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

68. The insulin derivative according to claim 54 which is in the form of a hexamer.

69. The insulin derivative according to claim 68, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

70. The insulin derivative according to claim 68, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, and Xaa at position B1 is Phe.

71. The insulin derivative according to claim 68, wherein two zinc ions bind to the hexamer.

72. The insulin derivative according to claim 71, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

73. The insulin derivative according to claim 68, wherein three zinc ions bind to the hexamer.

74. The insulin derivative according to claim 73, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

75. The insulin derivative according to claim 68, wherein four zinc ions bind to the hexamer.

76. The insulin derivative according to claim 75, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

77. A pharmaceutical composition which is an aqueous solution, comprising (a) an insulin derivative according to claim 54, (b) an isotonic agent, (c) a preservative and (d) a buffer.

78. The pharmaceutical composition according to claim 77, wherein the pH of the aqueous solution is in the range of 6.5–8.5.

79. The pharmaceutical composition according to claim 77, wherein the solubility of the insulin derivative exceeds 600 nmol/ml of the aqueous solution.

80. The pharmaceutical composition according to claim 77, further comprising an insulin or an insulin analogue which has a rapid onset of action.

81. The pharmaceutical composition according to claim 77, wherein the insulin derivative is a Zn^{2+} complex.

82. The pharmaceutical composition according to claim 77, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, and Xaa at position B1 is Phe.

83. The pharmaceutical composition according to claim 77, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

84. The pharmaceutical composition according to claim 77, wherein the insulin derivative is in the form of a hexamer.

85. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition according to claim 77.

86. The insulin derivative of claim 54, wherein the lipophilic substituent is cyclohexylvaleroyl.

87. The insulin derivative of claim 54, wherein the lipophilic substituent is acyl-glutamyl wherein the acyl is a linear, saturated acyl having 6 to 24 carbon atoms.

88. The insulin derivative of claim 54, wherein the lipophilic substituent is lauroyl.

89. The insulin derivative of claim 54, wherein the lipophilic substituent is myristoyl.

90. The insulin derivative of claim 54, wherein the lipophilic substituent is palmitoyl.

91. The insulin derivative of claim 54, wherein the lipophilic substituent is 2-succinylamido myristic acid.

92. The insulin derivative of claim 54, wherein the lipophilic substituent is 2-succinylamido palmitic acid.

93. The insulin derivative of claim 54, wherein the lipophilic substituent is 2-succinylamidoethoxy palmitic acid.

94. The insulin derivative of claim 54, wherein the lipophilic substituent is myristoyl- α -glutamyl.

95. The insulin derivative of claim 54, wherein the lipophilic substituent is myristoyl- α -glutamyl-glycyl.

96. The insulin derivative of claim 54, wherein the lipophilic substituent is choloyl.

97. The insulin derivative of claim 54, wherein the lipophilic substituent is 7-deoxycholoyl.

98. The insulin derivative of claim 54, wherein the lipophilic substituent is lithocholoyl.

99. The insulin derivative of claim 54, wherein the lipophilic substituent is lithocholoyl-glutamyl.

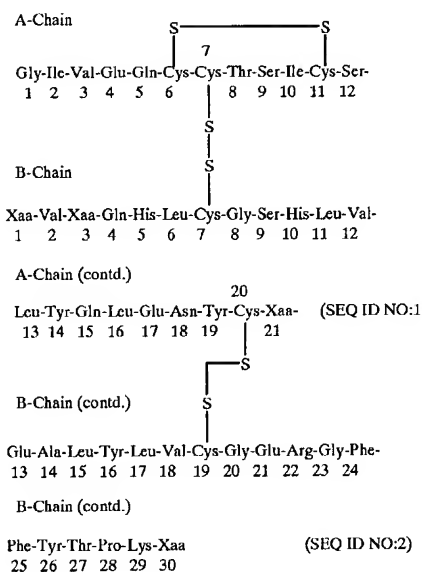
100. The insulin derivative of claim 54, wherein the lipophilic substituent is 4-benzoyl-phenylalanine.

101. The insulin derivative of claim 54, wherein the lipophilic substituent is L-thyroxy.

102. The insulin derivative of claim 54, wherein the lipophilic substituent is suberoyl-D-thyroxine.

103. The insulin derivative of claim 54, wherein the lipophilic substituent is 3,3',5,5'-tetraiodothyroacetyl.

104. An insulin derivative having the following sequence:



wherein

(a) Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

(b) Xaa at position B1 is Phe or is deleted;

(c) Xaa at position B30 is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; and

(d) the ϵ -amino group of Lys^{B29} is substituted with a lipophilic substituent having at least 10 carbon atoms; wherein the lipophilic substituent is benzoyl, phenylacetyl, cyclohexylacetyl, 3,5-diiodotyrosyl or cyclohexylpropionyl and the insulin derivative is a Zn^{2+} complex and the Zn^{2+} complex of the insulin derivative is more water soluble than the insulin derivative without Zn^{2+} .

105. The insulin derivative of claim 104, wherein the lipophilic substituent is benzoyl.

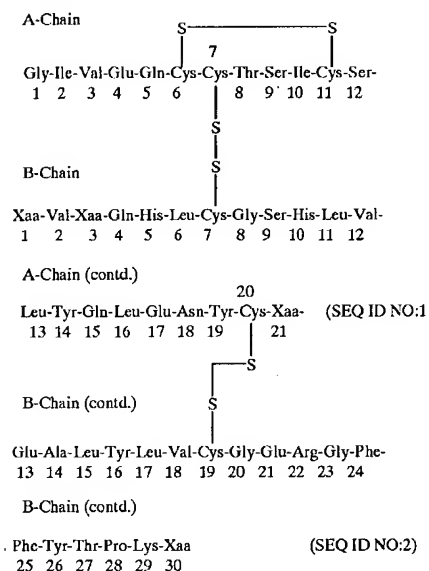
106. The insulin derivative of claim 104, wherein the lipophilic substituent is phenylacetyl.

107. The insulin derivative of claim 104, wherein the lipophilic substituent is cyclohexylacetyl.

108. The insulin derivative of claim 104, wherein the lipophilic substituent is 3,5-diiodotyrosyl.

109. The insulin derivative of claim 104, wherein the lipophilic substituent is cyclohexylpropionyl.

110. An insulin derivative having the following sequence:



wherein

- (a) Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;
 - (b) Xaa at position B1 is Phe or is deleted;
 - (c) Xaa at position B30 is deleted; and
 - (d) the ϵ -amino group of Lys^{B29} is substituted with a lipophilic substituent having at least 10 carbon atoms;
- wherein the lipophilic substituent is benzoyl, phenylacetyl, cyclohexylacetyl, 3,5-diiodotyrosyl or cyclohexylpropionyl and the insulin derivative is a Zn^{2+} complex and the Zn^{2+} complex of the insulin derivative is more water soluble than the insulin derivative without Zn^{2+} .
111. The insulin derivative of claim 110, wherein the lipophilic substituent is benzoyl.
 112. The insulin derivative of claim 110, wherein the lipophilic substituent is phenylacetyl.
 113. The insulin derivative of claim 110, wherein the lipophilic substituent is cyclohexylacetyl.
 114. The insulin derivative of claim 110, wherein the lipophilic substituent is 3,5-diiodotyrosyl.
 115. The insulin derivative of claim 110, wherein the lipophilic substituent is cyclohexylpropionyl.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,011,007
DATED : January 4, 2000
INVENTOR(S) : Havelund et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page.

Item [62], **Related U.S. Application Data**, please delete "Continuation-in-part of application No. 08/400,256, March 8, 1995, U.S. Pat. No. 5,750,497, which is a continuation-in-part of application No. 08/190,829, filed as application No. PCT/DK94/00347, Sep. 16, 1994, abandoned"

and insert

-- Continuation-in-part of application No. 08/400,256, filed March 8, 1995, U.S. Pat. No. 5,750,497, which is a continuation-in-part of application No. 08/190,829, filed Feb. 2, 1994, now abandoned, and application No. PCT/DK94/00347, filed Sep. 16, 1994, now abandoned --.


Column 1.

Line 7, please delete "5,750,997" and insert -- 5,750,497 --.

Signed and Sealed this

Twenty-sixth Day of November, 2002

Attest:



Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

Organization TC1600 Bldg./Room REMPSEN

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